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INVERSIONS-HETEROZYGOTIE UND FERTILITÄT DER MÄNNCHEN VON CHIRONOMUS

Von

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Mit 7 Textabbildungen

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Inversions-Polymorphismus ist bei den Dipteren weit verbreitet (vgl. DA CUNHA 1955). Sein Vorkommen wird im allgemeinen als Heterosis-Effekt interpretiert: Die Inversions-Heterozygoten sollen gegenüber den Homozygoten Selektionsvorteile besitzen. Der relative Selektionswert eines Genotypus hängt von der Vitalität und von der Fertilität des durch ihn bestimmten Phänotypus ab. In der Erörterung der Inversions-Heterosis hat bisher die Frage der relativen Vitalität im Vordergrund gestanden. Das Problem der relativen Fertilität der Heterozygoten dagegen konnte vorläufig vernachlässigt werden, weil die Chromosomen der hauptsächlich untersuchten Gattungen *Drosophila* und *Sciara* in der ♂-Meiose keine Chiasmen bilden. In der ♀-Meiose können Chromatidenbrücken (durch einfachen Austausch im Bereich heterozygoter Inversionen) zwar entstehen, doch erhält der präsumptive weibliche Vorkern in der Anaphase II bekanntlich stets eine der beiden nicht am Austausch beteiligten Chromatiden zugeteilt (STURTEVANT und BEADLE 1936; CARSON 1946); die Fertilität der Weibchen wird durch Inversions-Heterozygotie also nicht beeinträchtigt.

Drosophila und *Sciara* verkörpern den achiasmatischen Typus der Spermatogenese: bei vielen Nematoceren sind dagegen die meiotischen Bivalente auch im männlichen Geschlecht chiasmatisch gepaart, so z.B. bei der Mehrzahl der Tipuliden, den Limoniiden, Chironomiden und Culiciden. In diesen Gruppen muß die Entstehung von Brücken und Fragmenten durch crossing-over in heterozygoten Inversionen dementsprechend auch in der Spermatogenese erwartet werden. In der Tat hat WOLF (1941) Brücken mit Fragment in fast jeder Anaphase I von mehreren Männchen der Mücke *Dicranomyia trinotata* gefunden und ihr Verhalten ausführlich dargestellt (s. unten). Die Frage, welchen möglichen Einfluß die Bildung solcher Brücken auf die Fertilität der ♂♂ von *Chironomus* ausübt, ist von PHILIP (1942) erörtert worden, und zwar im Hinblick auf die weite Verbreitung des Inversions-Polymorphismus gerade bei den Chironomiden. PHILIP meint, möglicherweise

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seien Spermien mit aberrantem Chromosomenbestand, wie sie nach Brückenbildung entstehen müßten, den normalen gegenüber bei der Besamung und Befruchtung der Eier im Nachteil — obwohl man natürlich ebensogut an eine einfache Kompensation des Fertilitätsverlustes der Heterozygoten durch erhöhte Vitalität denken könnte, um die Tatsache der Inversions-Heterosis zu verstehen. Zudem blieb vorläufig noch die Möglichkeit offen, daß die Chiasmen in der Spermatogenese der Chironomus-♂♂ gesetzmäßig stets außerhalb der Inversionen gebildet würden, also trotz chiasmatischer Meiose Brücken nicht entstünden. Inzwischen sind aber die entsprechenden meiotischen Bilder bei *Chironomus dorsalis* (ACTON 1956) und *Chironomus tentans* (Abb. 1) in großer Anzahl gefunden worden, so daß die letztgenannte Deutung entfällt. Es bleibt deshalb weiterhin ein Problem, ob und bis zu welchem Grade die regelmäßige Bildung der dikinetischen Brücken in der Spermatogenese die männliche Fertilität herabmindern kann.

Abb. 1. Dikinetische Brücken mit akinetischem Fragment in der Anaphase I der Spermatogenese von *C. tentans*. Die Brücken sind durch Austausch innerhalb der heterozygoten Inversion *In 4—1* des telokinetischen kurzen Chromosoms 4 entstanden. Brücke und Fragment in den Zeichnungen schwarz, die beiden normalen Chromatiden punktiert. Orcein-Eisessig-Milchsäure (OEM)

Chironomus tentans besitzt haploid 4 Chromosomen, 3 lange mediokinetische und 1 kurzes, nahezu telokinetisches. In allen Chromosomenarmen kommen Inversionen vor (vgl. BEERMANN 1955). Zur Durchführung der Untersuchung erweist sich die Inversion *In(t)4—1* (im folgenden abgekürzt als „*In4*") des kurzen 4. Chromosoms als am besten geeignet (eine genauere Beschreibung dieser Inversion findet sich bei BAUER und BEERMANN 1952 und BEERMANN 1955): Das meiotische Bivalent 4 ist von den anderen 3 Bivalenten infolge seiner geringeren Größe leicht und sicher zu unterscheiden: eine Verwechslung der *In4*-Brücken mit Brücken, die auf andere Inversionen zurückgehen, kommt daher nicht in Frage. Zudem ist *In4* fast so lang wie das ganze Chromosom; damit wird die Bildung von Chiasmen außerhalb der Inversion sehr unwahrscheinlich (vgl. Tabelle 1). Schließlich läßt sich dem Bivalent 4 bereits in der Prometaphase I mit Bestimmtheit ansehen, ob das Chiasma innerhalb der Inversion gebildet worden ist oder nicht (Abb. 2 und 3); man ist bei der Statistik also nicht auf die seltenen Anaphasefiguren angewiesen. An sich muß einfacher Austausch in jeder anderen einfachen Inversion ebenfalls die in Abb. 2 und 3

dargestellten, teilweise asymmetrischen Konfigurationen ergeben (vgl. DARLINGTON 1937, Fig. 9), doch wird die Asymmetrie nur bei langen Inversionen in telokinetischen Chromosomen deutlich und ist deshalb bisher kaum beobachtet worden[1].

a b c d

Abb. 2a—d. Prometaphasische Bivalente des 4. Chromosoms von *C. tentans* bei Heterozygotie für *In 4—1*. a, b, c Ganze Prometaphasen mit dem 4. Bivalent (stets am Rande der Platte) in der typischen Gestalt, die durch Bildung eines Chiasma innerhalb der Inversion zustande kommt; in d einzelne Typen von heterozygoten Inversions-Bivalenten des 4. Chromosoms. OEM, 2000fach

Untersucht wurden die Gonaden und gleichzeitig die Speicheldrüsen von männlichen Vorpuppen (Larven, die kurz vor der Verpuppung stehen), und zwar von Geschwistertieren aus einer Einzelkreuzung: *In4/In4* ♀ × *In4/St* ♂ (*St* = Standard-Genanordnung des 4. Chromosoms). Neben *In4* kamen in den anderen Chromosomen dieser Tiere stets die komplexe „Y"-Inversion *In(t)1—k1* (vgl. BEERMANN 1955) und gelegentlich auch die einfache Inversion *In(t) 3L—1* heterozygot vor; beide können das Ergebnis der Untersuchung nicht beeinflussen.

Wie aus der Tabelle 1 klar hervorgeht, hat die Heterozygotie für *In4* kein Absinken der Chiasmenhäufigkeit zur Folge. Sowohl bei Hetero- als auch bei Homozygoten *(In4/In4)* wurden univalente Chromosomen 4 in nur 5 % bis 10 % der Meiosen angetroffen; aus Einzelbeobachtungen an anderem Material (s. auch Tabelle 1) ist allerdings bekannt, daß dieser Wert ausnahmsweise — aber unabhängig von der Inversions-Heterozygotie — bis auf fast 50% anwachsen kann (durch allgemeine

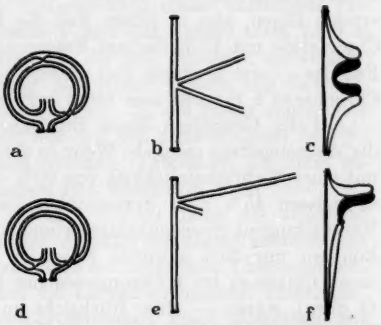


Abb. 3 a—f. Schematische Darstellung des Zustandekommens der in Abb. 2 gezeigten Formen von Bivalenten des Chromosoms 4. a, b, c Chiasma in der Mitte des invertierten Bereiches; d, e, f Chiasma an der Grenze der Inversion. a, d Pachytän; b, e Übergang vom Diplotän zur Prometaphase (eine Diakinese gibt es bei Chironomus nicht); c, f Prometaphase. Brücke und Fragment schwarz

[1] *Anmerkung bei der Korrektur:* Die Bildung von asymmetrischen Diakinese-Bivalenten durch Inversions-Chiasmata bei *Lilium* wird in einer soeben erschienenen Arbeit von BROWN und ZOHARY (1955) beschrieben und diskutiert.

Tabelle 1. *Häufigkeit der Chiasmen im Bivalent 4 von Chironomus tentans bei Inversions-Heterozygotie und -Homozygotie*

Bezeichnung des Individuums[1]	Konstitution	Metaphasen mit einem Chiasma im Bivalent 4		Metaphasen ohne Chiasma im Chr. 4	Chiasmenfrequenz im Bivalent 4
		innerhalb der Inversion	außerhalb der Inversion		
S 1	$\frac{In\,4-1}{st}$	89	0	11	0,89
S 31		93	0	7	0,93
S 34		29	0	1	0,97
S 38		94	0	6	0,94
S 40		90	0	10	0,90
S 43		100	0	7	0,94
S 44		93	0	7	0,93
SP 1/1		85	0	6	0,81
S 2	$\frac{In\,4-1}{In\,4-1}$	86		14	0,86
S 12		96		4	0,96
S 21		90		10	0,90
41 C2	$\frac{st-4}{st-4}$	53		47	0,53

[1] S = Stamm Schweden; SP 1/1 = F_2 von Schweden × Plön; 41 C2 = vor mehreren Jahren gehaltener Plöner Stamm.

Senkung der Chiasmafrequenz in allen Chromosomen). Zum zweiten zeigt die Meiosestatistik, daß sämtliche im inversions-heterozygoten 4. Bivalent gebildeten Chiasmen erwartungsgemäß innerhalb der Inversion liegen, also in jedem Fall die Entstehung einer dikinetischen Chromatide mit akinetischem Fragment zur Folge haben (der seltene Fall des — erst in einem Fall nachgewiesenen — Doppelaustauschs im Chromosom 4 braucht hier nicht berücksichtigt zu werden).

Auf der Grundlage dieser Ergebnisse wird eine präzisere Fassung der Ausgangsfrage möglich: Wenn in den Meiosen heterozygoter *In 4*-♂♂ mit einer Wahrscheinlichkeit von 90% dikinetische Brücken entstehen, so müssen 45% aller Spermatiden (bei sonst normalem Ablauf der Reifeteilungen) einen unbalancierten Chromosomenbestand erhalten; es könnten nur 55% normale Spermien gebildet werden. Und selbst, wenn Chiasmen im 4. Chromosom nur in 50% aller Meiosen aufträten (s. oben), wären — ohne Rücksicht auf das möglicherweise aberrante Verhalten der Univalenten — noch 25% Gameten mit nicht balanciertem Genom zu erwarten. Würden sich also die chromosomal aberranten Spermatiden in gleicher Weise wie die euploiden zu voll funktionstüchtigen Spermien differenzieren, so dürfte man unter den Nachkommen eines heterozygoten *In 4*-♂ 25—50% chromosomal nicht balancierte und daher wahrscheinlich subvitale Zygoten erwarten.

Nachdem in vergangenen Jahren bei Untersuchungen mit anderem Ziel immer wieder (in mindestens 20 Fällen) beobachtet worden war, daß die F_1-Embryonen von heterozygoten *In 4*-♂♂ zu mehr als 95%

Tabelle 2. *Prozentuale Häufigkeit und cytologische Konstitution der normal entwickelten Individuen aus Kreuzungen mit inversions-heterozygotem (In4—1) Vater*

Kreuzung[1] ♂♂ stets $\frac{In\,4-1}{st}$	Normal entwickelte und geschlüpfte Eilarven[2]	Anzahl der zur Aufzucht angesetzten Larven	Überlebende Larven					
			Anzahl im Stadium		Cytologische Konstitution			
			II - III	III - IV	Anzahl der untersuchten Larven	$\frac{In4-1}{st}$	$\frac{st}{st}$ oder $\frac{In4-1}{In4-1}$	Haplo-4 bzw. Brücke
1. SP5 ♀ × SP6 ♂	> 99%	100		71	0	—	—	—
2. SP5 ♀ × SP6 ♂[3]	~ 20%[4]	160		105	96	46[5]	50	0
3. EES ♀ × SP6 ♂	> 90%	50		47	0	—	—	—
4. EES ♀ × SP6 ♂	> 99%	100	90[6]	—	—	—	—	—
		100		82	82	40	42	0

[1] E = Stamm England, weitere Abkürzungen s. Fußnote Tabelle 1; die ♀♀ in Kreuzung 1 und 2 sind homozygot *st*, die ♀♀ in Kreuzung 3 und 4 homozygot *In4—1*.

[2] Anzahl der durchschnittlich in einem Gelege vorhandenen Eier ~1500.

[3] Gleiches ♂ wie in Kreuzung 1.

[4] Rest unbefruchtet, vgl. Text.

[5] Darunter 7 Triplo-4-Individuen, deren Vorkommen mit der Inversion in keinem Zusammenhang steht.

[6] Zucht nicht weitergeführt.

eine normale Entwicklung durchlaufen und alle gleichzeitig schlüpfen, wurde kürzlich mit der näheren Untersuchung dieser auffälligen Tatsache begonnen. Jedes *Chironomus*-♀ legt (im Labor) etwa 1500 in einer Gallertmasse vereinigter Eier ab, die sich synchron entwickeln; das Chorion ist völlig durchsichtig, und daher können alle Entwicklungsstörungen unmittelbar erkannt werden. In den bisher angesetzten Einzelpaarungen strukturhomozygoter ♀♀ mit heterozygoten *In4*-♂♂ bestätigten sich die älteren Beobachtungen (Tabelle 2); die nicht geschlüpften Eier im Versuch 2 hatten sich überhaupt nicht entwickelt — keine Polzellenabschnürung — und müssen daher als unbesamt angesehen werden. Um nun zu prüfen, ob unter den normal entwickelten und geschlüpften Embryonen etwa solche mit abnormem Chromosomenbestand vorhanden waren, wurden die Larven cytologisch untersucht. Die Analyse der Speicheldrüsen-Chromosomen kann allerdings mit Erfolg erst gegen Ende des 3. Larvenstadiums und im 4. Stadium durchgeführt werden; deshalb mußte die Möglichkeit ausgeschlossen werden, daß die etwa vorhandenen Individuen mit unbalanciertem Genom regelmäßig in der Jugend absterben[1]. Dazu mußten begrenzte

[1] Zumindest für den Haplo-4-Genotypus kann trotz der geringen relativen Länge des Chromosoms 4 mit dieser Möglichkeit gerechnet werden, da Haplo-4-Individuen in zahlreichen Versuchen mit *C. tentans* und *C. pallidivittatus* (über 3000 Speicheldrüsenpräparate) noch nie gefunden wurden, im Gegensatz zu immer wieder vereinzelt auftretenden Triplo-4-Tieren (vgl. auch Anm. 4 der Tabelle 2).

Mengen von frisch geschlüpften Larven zur Aufzucht angesetzt und nach dem Heranwachsen erneut abgezählt werden (vgl. Tabelle 2). Normalerweise ist die Sterblichkeit in *Chironomus*-Kulturen infolge unkontrollierbarer Bakterien- und Protozoenentwicklung ziemlich hoch; deshalb haben die 3 Versuche, in denen es bisher gelang, über 80% der angesetzten Larven großzuziehen, besonderes Gewicht.

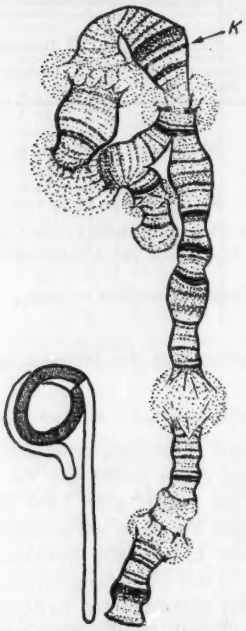


Abb. 4. Überzähliges dikinetisches 4. Chromosom in der Speicheldrüse eines F_2-Individuums der Kreuzung *C. tentans* × *C. pallidivittatus*. Das Brückenchromosom (im Schema punktiert; zu seiner Entstehung vgl. Text) ist lateral mit einem 4. Chromosom von *C. pallidivittatus* und außerdem mit dem proximalen Bereich eines invertierten 4. Chromosoms von *C. tentans* gepaart. In der Kinetochorregion (K) terminale Paarung (vgl. BAUER und BEERMANN 1952). Karmin-Essigsäure, etwa 900fach

Die cytologische Untersuchung der herangewachsenen Larven muß sich in erster Linie auf das Vorkommen von „Haplo-4"-Individuen richten; denn dikinetische Brücken oder ihre eventuellen Zerreißungsprodukte sind mitotisch instabil und könnten im Laufe der Furchungsteilungen oder später eliminiert worden sein.

In diesem Zusammenhang ist ein eigenartiger, älterer Einzelbefund zu erwähnen: Unter vielen Hunderten von cytologisch analysierten F_2-Tieren der Artkreuzung *C. tentans* × *C. pallidivittatus* (vgl. BEERMANN 1955) fand sich einmal eine Larve, deren Speicheldrüsenkerne zum Teil außer dem normalen, aus Chromosomen der beiden Arten kombinierten diploiden Chromosomenbestand zusätzlich ein dikinetisches Chromosom 4 aufwiesen (Abb. 4). Das „Brücken-Chromosom" muß durch einfaches crossing-over zwischen einem *In 4*-Chromosom von *C. tentans* und dem nicht invertierten homologen Abschnitt des Chromosom 4 von *C. pallidivittatus* entstanden sein; ob das dikinetische Chromosom mit dem weiblichen oder dem männlichen Vorkern in die Zygote gekommen ist, kann nicht entschieden werden. Unklar bleibt vor allem, auf welche Weise die Brücke als überzähliges Element in einen euploiden Sperma- oder Eikern gelangen konnte. Außerdem ist merkwürdig, daß die Brücke sich über mehrere Mitoseschritte hinweg — bis zur Anlage der Speicheldrüsen im Embryo — hat halten können. Die Tatsache, daß nur etwa die Hälfte aller Speicheldrüsenzellen in dem betreffenden Tier die Brücke besaßen,

zeigt immerhin, daß die Elimination als solche tatsächlich vorkommt und eine Rolle spielt.

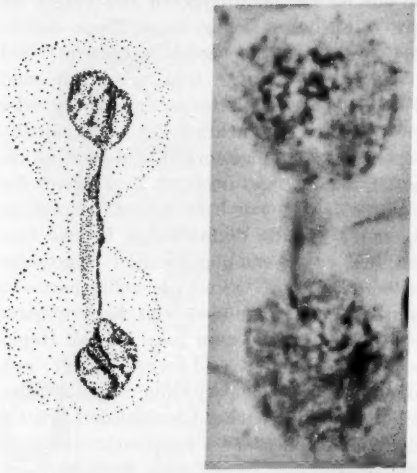
Die cytologische Analyse der F_1 von heterozygoten *In 4*-Vätern ist bisher erst bei 2 Kreuzungen in der oben angegebenen Weise durchgeführt worden; die Befunde (die Versuche werden fortgesetzt) sind eindeutig (Tabelle 2): Der Haplo-4-Genotypus tritt nicht auf, und ebensowenig werden Individuen mit der dikinetischen Brücke oder ihren Derivaten gefunden. Da bei den Aufzuchtversuchen im günstigsten Fall nicht einmal 10% der Zygoten abgestorben sind, und da die Sterblichkeit auch sonst unter dem Erwartungswert liegt, kann der Ausgang der cytologischen Untersuchung nur bedeuten, daß Spermien ohne 4. Chromosom und Spermien mit der dikinetischen Brücke bzw. deren Zerreißungsprodukten nicht zur Besamung der Eier beigetragen haben, ein Schluß, der ja auch schon auf Grund des völlig normalen Entwicklungsverhaltens sämtlicher Nachkommen von heterozygoten *In 4*-♂♂ hätte gezogen werden können, wenn man bedenkt, daß Haplo-4-Individuen als Zeichen ihrer verminderten Lebensfähigkeit wohl mindestens eine Verzögerung der Embryonalentwicklung zeigen müßten.

Wolf (1941) hat das Verhalten dikinetischer Chromatiden-Brücken in der Spermatogenese von *Dicranomyia* genauer untersucht und dabei Beobachtungen gemacht, die zu dem hier formulierten Problem in unmittelbarer Beziehung stehen:

> Bei *Dicranomyia* werden von verschiedenen Inversionen verschieden lange Brücken gebildet. Interessant ist in erster Linie das Verhalten der längsten Brücken. Sofern sie nicht zerreißen, was in der Mehrzahl der Fälle nicht der Fall zu sein scheint, bleiben sie während der Interkinese zwischen den Tochterkernen der I. Reifeteilung ausgespannt. Die beiden Kinetochoren der Brücke wandern in der Anaphase II ihrer metaphasischen Einstellung gemäß, jeder für sich in einer anderen Spindel, zu den Polen, so daß die Brücke schließlich zwischen 2 der 4 Gametenkerne ausgespannt erscheint. Wolf konnte zeigen, daß die beiden durch eine Brücke verbundenen Spermatiden verschmelzen und zu doppelkernigen Spermien werden. Andere Beobachtungen (an kürzeren Brücken) bestätigen die Vermutung, daß ganz allgemein die Durchschnürung des Cytoplasma bzw. des Spindelrestkörpers („Zellkoppel") unterbleibt oder nicht vollständig ist, wenn zwischen den Tochterkernen Chromatidenbrücken ausgespannt sind.

Bei *Chironomus* ist die Spermiohistogenese nicht so klar zu überblicken wie bei *Dicranomyia* — das gilt besonders für die Analyse des Chromosomenbestandes im Spermienkern. Doch sprechen die bisherigen Beobachtungen an *Chironomus* für eine weitgehende Übereinstimmung mit *Dicranomyia*, sowohl im Verhalten der langen Chromatidenbrücken als auch der Fragmente. Die aus heterozygoten *In 4*-Bivalenten durch crossing-over entstandenen langen Brücken verhindern die Bildung zweier getrennter Interkinesekerne nicht; die Brücken werden zwischen diesen Kernen ausgespannt (Abb. 5). Die beiden Spermatocyten II bleiben durch Zellkoppeln verbunden. In der Anaphase II erhält man

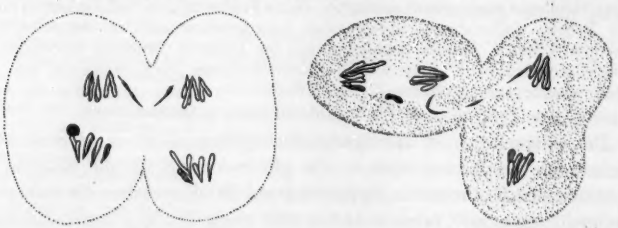
die gleichen Bilder wie bei *Dicranomyia*: Je zwei der Spermatiden sind durch eine Chromosomen- und eine Cytoplasmabrücke (Spindelrest der I.RT?) verbunden (Abb. 6). In der Telophase II werden die Brücken unsichtbar. Über das weitere Schicksal der aneinander gekoppelten Spermatiden fehlen noch genauere Daten: Doppelkernige Spermien sind beobachtet worden; ob diese aber auch 2 Schwänze ausbilden (s. unten), bleibt zu prüfen.



a b

Abb. 5 a u. b. Beginnende (a) und erreichte (b) Interkinese nach Bildung einer dikinetischen Brücke mit Fragment in der Spermatogenese von *C. tentans*. In a liegt das Fragment der Brücke oben an, in b sind beide nicht mehr zu sehen. Auf beiden Bildern erkennt man die aus dem Spindelrest gebildete Zellkoppel. OEM, 2000fach

Wie dem auch sei, die Untersuchungen an der F_1 von heterozygoten *In 4-♂♂* von *Chironomus tentans* zeigen bereits jetzt, daß die aus Doppelspermatiden eventuell entstehenden aberranten Spermien keine Besamungs-Chancen haben. Die morphologischen bzw. physiologischen Ursachen dieser Erscheinung müssen noch aufgeklärt werden. Die Zusammen-



a b

Abb. 6 a u. b. Anaphase II der Spermatogenese von *C. tentans* mit aus der I. meiotischen Teilung stammender dikinetischer Brücke und Fragment des 4. Chromosoms. Fragment und Brücke schwarz, die beiden normalen Chromatiden punktiert. In b Darstellung von hellen Plasmahöfen um die Anaphasegruppen und die Brücke; im Fall der Brücke handelt es sich vielleicht um Spindelüberreste der I. Reifeteilung. OEM, 2000fach

setzung des Genoms kann die Beweglichkeit und Leistungsfähigkeit von tierischen Spermien nicht beeinträchtigen, wie umfangreiche

genetische Erfahrungen mit Deficiencies und Duplikationen zeigen; möglich wäre es immerhin, daß Hyper- und Hypoploidie größeren Ausmaßes die Zeitdauer der Ausdifferenzierung der Spermatiden beeinflussen könnte, und daß das Sperma junger ♂♂ deshalb zunächst überwiegend normale Spermien enthielte. Viel näherliegend ist jedoch die oben schon angedeutete Möglichkeit, daß Doppel- oder Mißbildungen des Bewegungsapparates — vielleicht hervorgerufen durch das Vorhandensein zweier Centrosomen in der gleichen Spermatide — die Untauglichkeit der Doppelspermien bedingen. Es konnte beobachtet

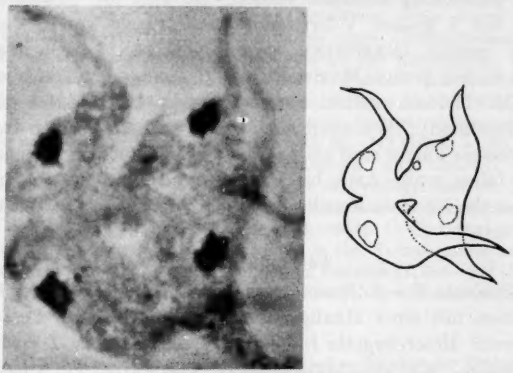


Abb. 7. Spermatiden-Tetrade aus einem heterozygoten *In 4*-Männchen von *C. tentans*. In der Zeichnung sind die Umrisse der auswachsenden Spermienschwänze und die Lage des Fragments verdeutlicht. Die Brücke war nicht zu erkennen. OEM, 2000fach

werden, daß eine Spermatiden-Tetrade mit 4 Kernen und einem Fragment (also auch mit einer möglicherweise unzerrissenen Brücke) 4 getrennte Bewegungsapparate ausbildete (Abb. 7). Weitere Untersuchungen müssen zeigen, ob eine solche Deutung das Richtige trifft. Jedenfalls ist durch diese und WOLFs Befunde wahrscheinlich gemacht worden, daß die inversions-heterozygoten Männchen auf ganz ähnliche Weise wie ihre weiblichen Artgenossen stets nur normale euploide Gameten produzieren, obwohl in der Meiose regelmäßig Inversionsbrücken entstehen. Für *Dicranomyia* ist dieser Schluß schon von BAUER und TIMOFÉEFF-RESSOVSKY (1943) gezogen worden.

Formal gesehen, sind inversions-heterozygote ♂♂ allerdings auch dann nicht uneingeschränkt fertil, wenn die oben geäußerten Schlußfolgerungen zutreffen; denn auf keinen Fall kann (ceteris paribus) die absolute Anzahl der von inversions-heterozygoten ♂♂ produzierten funktionstüchtigen Spermien die gleiche sein wie die von strukturell homozygoten ♂♂. Dieser Unterschied kann jedoch deshalb kaum eine Rolle spielen, weil die von einem *Chironomus*-♂ produzierte Anzahl von

Spermien sicher weit über 10000 beträgt, von denen im Durchschnitt höchstens 3000 zur Besamung verwendet werden (unter der Voraussetzung, daß jedes ♂ nur einmal kopuliert, jedes ♀ höchstens 3000 Eier ablegt und Polyspermie nicht vorkommt)[1]. Der Selektionswert inversions-heterozygoter Männchen wird also durch die theoretisch zu fordernde zahlenmäßige Einbuße an normalen Spermien kaum beeinträchtigt. Erst wenn mehrere Inversionen in verschiedenen Chromosomen vorlägen, könnte sich dies ändern: Jede zusätzliche Brücke erhöht ja die Wahrscheinlichkeit, daß alle 4 Partner einer Spermatiden-Tetrade gleichzeitig aberrant werden. Da aber die Chiasmenfrequenz je Arm der 3 großen V-förmigen Chromosomen von *C. tentans* nur 0,4—0,5 beträgt (BEERMANN unveröffentlicht), können Inversionsbrücken in den großen Bivalenten von *C. tentans* ohnehin höchstens in 50% aller Meiosen auftreten (sofern nicht beide Arme inversionsheterozygot sind). Es kommt noch hinzu, daß die meisten Inversionen von *C. tentans* kürzer sind als *In 4*, so daß nicht jedes Chiasma in ihren Bereich fallen muß. Auch bei Heterozygotie für 3 oder 4 Inversionen wird deshalb die reale Fertilität der ♂♂ von *Chironomus* kaum herabgesetzt sein[2].

Zusammenfassung

1. Bivalente des 4. Chromosoms von *C. tentans* bilden in der Spermatogenese mit einer Häufigkeit von bis zu 95% ein Chiasma auch dann, wenn Heterozygotie für die Inversion *In(t)4—1* vorliegt.

2. Bereits den prometaphasischen Bivalenten des Chromosoms 4 läßt sich mit Sicherheit ansehen, daß bei Heterozygotie für *In(t)4—1* das Chiasma stets innerhalb der Inversion entsteht —, daß demnach in bis zu 95% aller Meiosen eines Männchen eine dikinetische Chromatidenbrücke mit akinetischem Fragment gebildet wird.

3. Kreuzungsversuche zeigen — in Verbindung mit kontrollierter Aufzucht und cytologischer Analyse — daß inversions-heterozygote ♂♂ *(In4—1)* eine voll lebensfähige und cytologisch normale F_1 ergeben.

4. Die Befunde lassen sich am besten so deuten, daß die durch eine Chromatidenbrücke verbundenen Spermatiden in der Spermiohistogenese ganz oder teilweise miteinander verschmelzen, wie das bei einem

[1] Der Befund, daß in der Kreuzung 2 (Tabelle 2) etwa 80% der Eier unbefruchtet blieben, läßt sich vielleicht in diesem Sinne als Folge einer Erschöpfung des väterlichen Spermienvorrates deuten, wie sie eintreten muß, wenn ein- und dasselbe ♂ mehrere ♀♀ begattet; ob der Spermienvorrat eines heterozygoten *In4—*♂ allerdings nicht einmal für 2 Gelege (im Labor rund 4000 Eier) ausreicht, erscheint zweifelhaft und wird noch untersucht. Möglicherweise hatte das zu den Kreuzungen 1 und 2 benutzte ♂ schon vor seiner Isolation kopuliert.

[2] Die Untersuchungen werden fortgesetzt. Eine ausführliche Darstellung der Meiose der *Chironomus*-♂♂ ist in Vorbereitung.

anderen Objekt schon nachgewiesen ist (WOLF 1941). Die auf diese Weise entstandenen Doppelspermien sind vermutlich minderwertig und zur Besamung nicht fähig. Die Verringerung der absoluten Anzahl der normalen Spermien kann sich auf die Fertilität und damit den Selektionswert der inversions-heterozygoten Männchen in der Natur kaum auswirken.

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BEOBACHTUNGEN ÜBER DIE ♂-MEIOSE DER MUSCHEL SPHAERIUM CORNEUM

Von

H.-G. Keyl

Mit 8 Textabbildungen

(Eingegangen am 14. Januar 1956)

Der Karyologie der Keimzellenentwicklung bei den Lamellibranchiern ist bisher wenig Beachtung geschenkt worden, obwohl Eier und Spermien verschiedener Arten wiederholt als Objekte entwicklungsphysiologischer und cytologischer Untersuchungen gedient haben. Über das Chromosomen*verhalten* während der Meiose ist jedenfalls noch nichts bekannt. Die nur bei wenigen Arten festgestellten Chromosomenzahlen — *Cumingia tellinoides* n = 18 (Jordan 1910, Morris 1917), *Cumingia spec.* n = 16 (Mackay 1927), *Mactra spec.* n = 12 (Kostanecki 1904), *Ostrea ariakensis*; *O. laperousi*, beide 2n = 24 (Kobayashi 1954), *Unio spec.* n = 16 (Lillie 1901) — wurden ausschließlich in der Oogenese oder der frühen Embryonalentwicklung ermittelt.

Bei der Durchsicht der häufigsten einheimischen Muscheln auf ihre Eignung für die Analyse der Meiose erwies sich *Sphaerium corneum* als allein brauchbare Art. Hohe Chromosomenzahlen und sehr kleine Kerne machen bei Vertretern der Gattungen *Anodonta* und *Unio* und bei *Dreissensia polymorpha* eine befriedigende Analyse unmöglich. Ungünstig wirkt sich ferner die bei den meisten Arten zu beobachtende Fixierungslabilität der Chromosomen aus, die auch bei den marinen Muscheln verbreitet zu sein scheint. Die marinen Arten *Mya arenaria*, *Cardium edule* und *Mytilus edulis* sind wegen des Verklebens der Chromosomen bei der Fixierung für die cytologische Analyse der Meiose völlig ungeeignet[1].

Die zur Untersuchung benötigten Sphaerien wurden Anfang Juni aus einem Graben der Umgebung von Wilhelmshaven gesammelt. Die Hoden wurden herauspräpariert und nach Fixierung in Alkohol-Eisessig (3:1) mit Karminessigsäure gefärbt.

Befunde

Die verhältnismäßig großen Metaphaseplatten der primären Spermatogonien enthalten 36 medio- bzw. subtelokinetische Chromosomen (Abb. 1).

[1] Herrn Prof. Dr. H. Bauer danke ich für seine anregende Kritik, die er meinen Untersuchungen hat zuteil werden lassen.

Die Leptotän- und Pachytänstadien der Spermatocyten zeigen keine Abweichungen vom normalen Verhalten. Im frühen Diplotän treten die Spiralwindungen, mit denen sich die Homologen vereinigen (relational coiling), deutlich in Erscheinung (Abb. 2). Die folgenden Stadien sind schwierig zu analysieren, weil die Kerne zunächst von einem dichten Geflecht dünner Chromosomenfäden erfüllt werden, so daß man auf die Beobachtung einzelner günstig gelagerter Bivalente angewiesen ist.

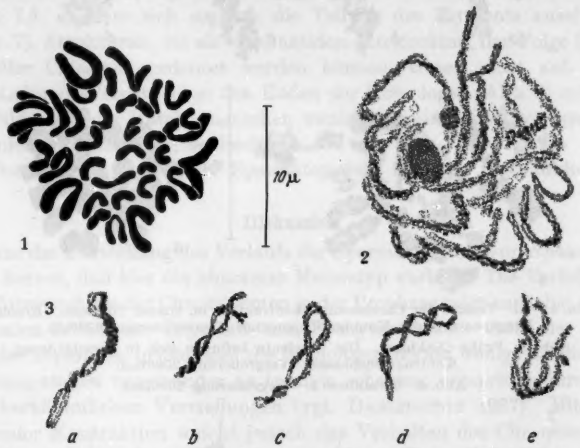

Abb. 1. Metaphaseplatte einer primären Spermatogonie. 2n = 36 Chromosomen. Vergrößerung 2000fach

Abb. 2. Chromosomen im Pachytänstadium. Vergrößerung 2000fach. (Nicht alle Chromosomen sind dargestellt)

Abb. 3a—e. Bivalententwicklung vom frühen bis mittleren Diplotän. Vergrößerung 2000fach

Die mit Beginn des Diplotän eingeleitete Lockerung des Kontakts zwischen den Konjugationspartnern schreitet weiter fort (Abb. 3). Dabei wird die Zahl der Umwindungen herabgesetzt, die Homologen wickeln sich also weitgehend voneinander ab. Über die Eigenschaften der verbleibenden Paarungsstellen läßt sich aus der Beobachtung nichts Eindeutiges aussagen. Zwar sind die Chromatiden meist über weite Strecken der Chromosomen identifizierbar, ihr Verlauf an den Paarungsstellen ist jedoch im allgemeinen nicht, bzw. nur in den Fällen zu erkennen, in denen es sich einwandfrei nicht um Chiasmen handelt (vgl. Abb. 3e). Die Kontraktion der Chromosomen erfolgt unter auffallend deutlicher Spiralisierung. Es ist bemerkenswert, daß die für das Diplotän bezeichnende Tendenz zur Aufhebung der Paarung von nun ab verschwindet und daß allmählich wieder eine verstärkte Annäherung

der Homologen eintritt (Abb. 4). Im Verlauf der Diakinese werden die noch sichtbaren Lücken zwischen den Partnerchromosomen völlig geschlossen; dabei glättet sich die Oberfläche der Bivalente (Abb. 5).

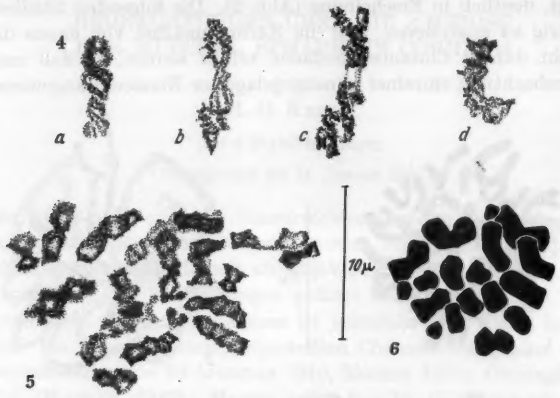

Abb. 4a—d. Verlauf der Chromosomenkontraktion im späten Diplotän. Erneute Längspaarung der Konjugationspartner. Vergrößerung 2000fach

Abb. 5. Frühe Diakinese. Die Bivalente befinden sich in verschiedenen Kontraktionsphasen. Vergrößerung 2000fach

Abb. 6. Metaphase I. Vergrößerung 2000fach

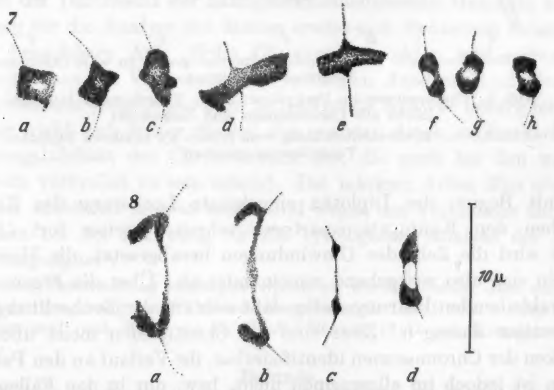

Abb. 7a—h. Bivalente bei beginnender Anaphase. Vergrößerung 2000fach

Abb. 8a—d. Partnertrennung in der mittleren Anaphase I. Vergrößerung 2000fach

Der mittlere Teil der Chromosomen, in dem das Kinetochor lokalisiert ist, wird in den Spiralisierungsprozeß zunächst nicht mit einbezogen (Abb. 4b, c). Während der Diakinese entsteht an dieser Stelle eine

Einschnürung (Abb. 5), die später im Zustand stärkster Kontraktion weitgehend schwindet, zweifellos aber für die V-Form der großen Metaphasechromosomen verantwortlich zu machen ist (Abb. 6). Dieser Verlauf der Prophase läßt mit Sicherheit erkennen, daß die Chromosomen in der Metaphase I aus Elementen bestehen, die über ihre ganze Länge miteinander gepaart sind.

Die Anaphase I beginnt mit dem Heraustreten der Kinetochoren (Abb. 7d, e), dem sich sogleich die Teilung des Bivalents anschließt (Abb. 7). Strukturen, die als Chromatiden-Interlocking, der Folge interstitieller Chiasmen gedeutet werden können, treten nicht auf. Die Verbindungsfäden zwischen den Enden der Homologen (Abb. 8) müssen als Bildungen der Matrix angesehen werden; sie sind auch bei Spermatogonien-Anaphasen zu beobachten.

Der weitere Verlauf der Spermatogenese ist ohne Besonderheiten.

Diskussion

Aus der Darstellung des Verlaufs der Spermatogenese von *Sphaerium* geht hervor, daß hier ein abnormer Meiosetyp vorliegt. Die Verfolgung des Formwechsels der Chromosomen in der Prophase zeigt zunächst einen normalen Ablauf der Leptotän- und Pachytänstadien. Auch der erste Teil des Diplotäns, in dem sich die Homologen bis auf wenige lokalisierte Paarungsstellen voneinander zu trennen scheinen, entspricht durchaus den herkömmlichen Vorstellungen (vgl. DARLINGTON 1937). Mit einsetzender Kontraktion weicht jedoch das Verhalten der Chromosomen ab: die sich spiralisierenden Elemente legen sich allmählich wieder aneinander und gewinnen in der Diakinese Kontakt in ihrer ganzen Länge. Hieraus resultiert die ungewöhnliche Form der Metaphasebivalente, die aus völlig längs gepaarten Chromosomen bestehen. Diese Erscheinung schließt die Annahme einer üblichen Terminalisation der über das ganze Bivalent verteilten und zunächst als Chiasmen anzusehenden Paarungsstellen aus. Unter diesen Voraussetzungen müßten die Chiasmen bis zur Anaphase an ihrem Bildungsort erhalten bleiben. Das Studium der Anaphasechromosomen gibt jedoch keinen Anhaltspunkt dafür, da die Homologen sich ohne jedes Hindernis voneinander lösen. Es besteht also Grund zu der Annahme, daß die Paarungsstellen im Diplotän keine echten Chiasmen sind, sondern lediglich nicht abgewickelte Spiralwindungen. Damit läge bei *Sphaerium* ein weiteres Beispiel des achiasmatischen Meiosetyps vor, ein Befund, der deshalb an Interesse gewinnt, weil unter den Mollusken nur in einem Fall, nämlich bei dem Prosobranchier *Bithynia tentaculata*, ein achiasmatischer Meioseverlauf wahrscheinlich gemacht werden konnte (KEYL 1955).

Da die Mollusken im allgemeinen wenig günstige Objekte für Chromosomenuntersuchungen sind, bleiben auch in der Meiose von *Sphaerium*

einige Fragen offen. So ist nicht zu klären, wie die letzten Umwindungen der Homologen, die in der Diakinese — offenbar unter der Tarnung der Matrix — verschwinden, abgewickelt werden. Dies könnte schon in den letzten Stadien der Kontraktion geschehen; vielleicht bleiben auch Windungen bis zur Anaphase erhalten und werden bei der Trennung der Partner aufgezogen. Gerade das Fehlen eindeutiger Beobachtungen in diesem Punkt könnte der Kritik die Möglichkeit geben, verdeckte Chiasmen zu postulieren, die sich unter den beschriebenen Umständen der Sicht entziehen. Ein einwandfreier Beweis für fehlende Chiasmen ist nur durch Vergleich mit Vererbungsversuchen zu erbringen, die jedoch entsprechend günstige Objekte erfordern und deshalb bisher nur in wenigen Fällen möglich gewesen sind [z. B. bei *Drosophila pseudoobscura* (Darlington 1934, Dobzhansky 1934); bei *Phryne fenestralis* (Bauer 1946, Wolf 1950)]. Ebenso könnte das Anaphaseverhalten der Bivalente bei künstlich erzeugten Inversionen als Beweismittel dienen. Diese Methoden sind jedoch aus technischen Gründen bei *Sphaerium* nicht anwendbar.

Von den bisher bekannten Beispielen achiasmatischer Meiosen, die bei Insekten und Protozoen beschrieben sind, dürften die meiotischen Vorgänge während der Pädogamie von *Actinophrys sol* (Bělař 1922) den Verhältnissen bei *Sphaerium* am ähnlichsten sein. Bauer (1946) und le Calvez (1950) haben darauf hingewiesen, daß bei den Voraussetzungen, wie sie durch die Abbildungen Bělařs (1922) dokumentiert sind, bei *Actinophrys* nur eine achiasmatische Meiose vorliegen kann.

Zusammenfassung

1. *Sphaerium corneum* hat 36 medio- bzw. subtelokinetische Chromosomen.

2. Die Meiose verläuft im männlichen Geschlecht bis zum mittleren Diplotän normal. Innerhalb der folgenden Kontraktionsphase treten die Homologen erneut zu einer Längspaarung zusammen. Eine Trennung der Partnerchromosomen erfolgt erst bei der Anaphase I.

3. Da unter diesen Umständen eine Terminalisation von Chiasmen nicht eintreten kann und Metaphase und Anaphase der ersten Reifungsteilung keine chiasmenähnlichen Strukturen erkennen lassen, wird vermutet, daß es sich um einen Fall von achiasmatischer Meiose handelt.

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DIE GEOGRAPHISCHE VERBREITUNG DER CHROMOSOMALEN STRUKTURTYPEN VON LIRIOMYZA UROPHORINA MIK.

Von

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Mit 1 Textabbildung

(Eingegangen am 11. Januar 1956)

Die vorliegende Untersuchung stellt eine Ergänzung und Erweiterung der von MAINX (1951) mitgeteilten Befunde dar. Es konnte durch die Prüfung von 1229 Larven von 29 neuen Standorten das damals untersuchte Gebiet besser erschlossen und darüber hinaus ein viel größerer geographischer Bereich erfaßt werden.

Die Feststellung, daß *Liriomyza* überall dort vorkommt, wo *Lilium martagon* ausgedehnte und dichtere Bestände bildet, konnte im allgemeinen bestätigt werden. Der Parasit fehlt meist dort, wo seine Wirtspflanze nur selten oder zerstreut auftritt, so an den Standorten Gmünd (N.Oe.), Ratschenhof bei Zwettl (N.Oe.), bei Dornbirn, Rankweil und Bezau im Bregenzer Wald (Vorarlberg), oder wo die lokalen Bestände von *Lilium martagon* keinen Zusammenhang mit anderen größeren Beständen zu haben scheinen, so bei Payerbach und Gloggnitz (Semmeringgebiet), bei Preinsfeld (südlicher Wiener Wald), bei Bruck a. d. Mur und Radkersburg (Steiermark) und bei Arriach (Kärnten). Die reichen Bestände von *Lilium martagon* bei Groß-Florian bei Deutschlandsberg (Steiermark) und bei Schärding am Inn (O.Oe.) sind ausgesprochene Isolate im betreffenden Gebiet und sind auch frei von Parasiten. Ob das gleiche auch für einen Standort bei Eichstätt (Bayern) gilt, der sich als parasitenfrei erwies, können wir nicht beurteilen. Es ist auffallend, daß die ganze nordwestliche Grenzzone des Verbreitungsgebietes von *Lilium martagon* in Deutschland von Parasiten frei zu sein scheint, obwohl es sich zum Teil um ausgedehnte und reiche Bestände der Pflanze handelt. In den uns wiederholt zugesandten Proben vom Hünstollen im Göttinger Wald, von Quast bei Rhoden und Eisenberg bei Korbach (Waldeck) und von Osterode (Harz) konnten keine Parasiten gefunden werden. Vielleicht hat der Parasit mit dem Vordringen der Pflanze in diese Gebiete nicht Schritt halten können. Einige der Standorte, die sich als parasitenfrei erwiesen, sind in der Kartenskizze (Abb. 1) bezeichnet.

Die Regel, daß der Parasit nur selten über 1000 m Seehöhe hinaufgeht, konnte für einige neue Standorte bestätigt werden (Navis in Tirol, 1600 m; Großer Pyhrgas, 1900 m; Plöckenpaß, 1360 m; Stockenboi in Kärnten, 1000 m; Hetzkogel bei Lunz am See, 1400 m). Doch hat diese Regel oft Ausnahmen. Von Parasiten befallen zeigten sich Proben vom Rauchkofel in den Lienzer Dolomiten (Osttirol) bei 1000 m, vom Raiblersee am Neveasattel (Kärnten) bei 1034 m, aus der Umgebung von Kufstein (Nordtirol) bei 1600 m und von Pettental bei Pusterwald (Bez. Judenburg, Steiermark) sogar bei 1750 m Seehöhe. Alle diese Standorte sind allerdings ziemlich warme Lagen, wo die Blütezeit von *Lilium martagon* keine zu starke Verschiebung gegen den Herbst zeigt (bei Kufstein Ende Juli 1952, bei Pettental schon Ende Juni 1951).

Zu den früheren cytologischen Angaben (MAINX, l. c.) sei das folgende hinzugefügt. Die Metaphase-Chromosomen in den Ganglien der Larven und in den Imaginalscheiben der Puppen sind zum größten Teil euchromatisch und zeigen die für Dipteren charakteristische Parallellagerung der Homologen. Außer dem kleinen, kugelförmigen Chromosomenpaar (Riesenchromosomen-Element VI) sind 4 ungefähr gleich lange, akrozentrische Paare vorhanden und ein etwas längeres akrozentrisches Paar, das dem Element I der Chromosomenkarte entsprechen dürfte. Ein Geschlechtsunterschied läßt sich weder in den somatischen Mitosen noch am Riesenchromosomensatz feststellen. Die Ruhekerne zeigen nur geringe Mengen von Heterochromatin, die dem schwach ausgebildeten Chromozentrum und den heterochromatischen proximalen Bereichen der Elemente I, III und V entsprechen dürften. Die in der Chromosomenkarte von MAINX (l. c.) dargestellte Querscheibenfolge des Elements II entspricht nicht der Standardanordnung, sondern der invertierten Anordnung. Um die Standardanordnung zu erhalten, müßte der eingeklammerte Bereich der Inversion II Ku umgekehrt werden. Als Standardanordnung soll für alle Chromosomen jener Strukturtyp gelten, der außerhalb der Verbreitungsgebiete der jeweiligen Inversionstypen allein homozygot vorhanden ist.

In den 1229 Larven der neu untersuchten Standorte wurden die von MAINX (l. c.) als weit verbreitet festgestellten Inversionstypen I Ku, II Ku, III Ku und V Sa mit wechselnder Häufigkeit wiedergefunden. Die damals nur je einmal gesehenen Typen IV Sa und V Bi wurden nicht wiedergefunden. Ein neuer Strukturtyp wurde nur in einem Material aus der Umgebung von Jena (Thüringen) entdeckt. Bei diesem tritt zur Inversion V Sa eine weitere, etwas längere, einfache Inversion hinzu. Ihre proximale Bruchstelle liegt nur 3 feine Querscheiben von der distalen Bruchstelle von V Sa entfernt, ihre distale Bruchstelle liegt noch 2 feine Querscheiben jenseits der beiden eng benachbarten Dubletten, zwischen denen in der Chromosomenkarte die proximale Bruchstelle der Inversion V Bi eingetragen ist. Diese neue Inversion liegt im gleichen Chromosom wie V Sa und ist wohl als phylogenetisch viel jünger anzusehen, da der Strukturtyp V Sa, wie noch berichtet werden soll, sehr weit verbreitet ist. Der neue Strukturtyp, der bisher nur in Jena gefunden wurde, soll im folgenden als V Je bezeichnet werden.

Die neuen Standorte, von denen Proben untersucht werden konnten, sind die folgenden (Fortsetzung der Numerierung von MAINX, l. c.): 12. Hollenburg a. d. Donau (1952). Der gleiche Standort wie 1. bei MAINX (l. c.). 13. Waldwiese bei Spitz a. d. Donau (N.Oe.) (1951), 550 m, linkes Donauufer. 14. Mischwald n.ö. von Stockerau (N.Oe.) (1952), 370 m. 15. Laubwald bei Hollabrunn (N.Oe.) (1951), 280 m. 16. Felshang bei Nieder-Fladnitz (N.Oe.) (1952), 400 m. 17. Mischwald

sü. von Falkenstein (N.Oe.) (1951), 320 m. 18. Buchenwald bei Gießhübl (Wienerwald) (1951), 620 m. 19. Buchenwald bei Mannersdorf im Leithagebirge (N.Oe.) (1951), 250 m. 20. Fichtenwald bei Gmunden (O.Oe.) (1951), 680 m. 21. Mischwald bei Sachrang (Oberbayern) (1952), 600 m. 22. Steilhang bei Kufstein (N.-Tirol) (1953), 1600 m. 23. Mischwald an der Westflanke des Rauchkofels bei Lienz (O.-Tirol) (1952/53), 1000 m. 24. Buchenwald-Steilhang sü. von Schloß Wernberg bei Villach (Kärnten) (1952), 520 m. 25. Fichtenbestand beim Raiblersee, Weg am Nevea-Sattel, sü. von Tarvis (Oberitalien, in der Nähe der Kärntner Grenze) (1951), 1035 m. 26. Fichtenwald und Schläge bei Unter-Loibl bei Ferlach (Kärnten) (1951—1953), 550 m. 27. Mischwald und Schlag bei St. Paul im Lavanttal (Kärnten) (1951), 500 m. 28. Almwiese Pettental bei Pusterwald, Bez. Judenburg (Steiermark) (1951), 1750 m. 29. Mischwald am Gollgeck, Bair. Kölldorf bei Gleichenberg (Steiermark) (1951), 390 m. 30. Fichtenwald am li. Ufer der Frutz bei Dornbirn (Vorarlberg) (1951), 450 m. 31. Buchenwald bei Heiligenberg, Kreis Ueberlingen (Bodensee) (1952), 500 m. 32. Fichtenwald Hakel bei Gatersleben (Prov. Sachsen) (1955), 220 m. 33. Berlin-Dahlem, Botanischer Garten (Parasit offenbar dorthin aus dem Spreewald verschleppt, wo Lilium martagon Bestände bildet) (1952). 34. Naumburg (Saale), Standort nicht näher bekannt (1952). 35. Laubgehölz an Steilhang bei Jena (Thüringen) (1954), 240 m. 36. Mischwald bei Margetshöchheim bei Würzburg (Franken) (1953), 200 m. 37. Mischwald bei Schloßberg Castell (Franken), etwa 40 km östlich von Standort 36 (1954), 400 m. 38. Reutlingen I, Buchenwald im „Sättele" zwischen Schönberg und Wackerstein, 5 km südlich von Reutlingen (Schwaben) (1954), 700 m. 39. Reutlingen II, nicht näher bekannter Standort südwestlich von Reutlingen (1954). 40. Freiburg i.B., Fichtenwald auf der „Wilden Steige" bei Ihringen im Kaiserstuhl, westlich von Freiburg i.B. (1954), 480 m.

In der Tabelle 1 sind die Befunde für alle bisher erfaßten Standorte zusammengestellt, wobei auch die Ergebnisse von Mainx (l. c.) einbezogen sind. Es sind die Heterozygotenfrequenzen der verbreiteten Inversionstypen I Ku, II Ku, III Ku und V Sa eingetragen. Wo die Zahl der Präparate zu gering war, ist das Vorkommen der Strukturtypen nur mit + (bei häufigem Vorkommen mit ++) bezeichnet, während über das Fehlen von Strukturtypen in diesen Fällen keine Aussage möglich ist. Bei genügender Präparatenzahl bedeutet das Zeichen — das Fehlen des betreffenden Inversionstyps. In der Kartenskizze (Abb. 1) sind die Standorte nach den Nummern dieser Tabelle aufzufinden.

Durch die Ausdehnung der Untersuchungen auf ein größeres geographisches Gebiet lassen sich die von Mainx (l. c.) aufgestellten Annahmen nachprüfen. Zunächst bestätigt es sich, daß die verschiedenen

Tabelle 1

Nr.	Standort	I Ku/St	II Ku/St	III Ku/St	V Sa/St	Zahl der Präparate
1	Hollenburg . . .	48 ± 9,0%	55 ± 9,0%	61 ± 8,8%	—	31
2	Kugelwiese . . .	23 ± 3,5%	37 ± 4,0%	5 ± 1,8%	—	142
3	Josefshöhe	36 ± 6,8%	42 ± 7,0%	28 ± 6,3%	—	50
4	Gaaden	—	56 ± 6,9%	—	—	52
5, 6	Lunz	—	48 ± 4,2%	22 ± 3,5%	10 ± 2,5%	144
7–9	Salzburg	—	41 ± 4,3%	23 ± 3,7%	2 ± 1,2%	132
10	Werfen	—	54 ± 7,6%	19 ± 6,0%	—	43
11	Bisamberg	43 ± 6,0%	—	15 ± 4,4%	—	67
12	Hollenburg . . .	49 ± 5,2%	45 ± 5,2%	40 ± 5,1%	—	92
13	Spitz	33 ± 8,2%	12 ± 5,6%	30 ± 8,0%	—	33
14	Stockerau	22 ± 5,6%	4 ± 2,6%	—	—	55
15	Hollabrunn . . .		+			3
16	Nieder-Fladnitz .	30 ± 11,0%	12 ± 7,9%	—	—	17
17	Falkenstein . . .	16 ± 5,9%	—	—	—	38
18	Gießhübl	4 ± 1,7%	25 ± 4,1%	30 ± 4,3%	—	114
19	Leithagebirge . .	41 ± 4,6%	18 ± 3,6%	18 ± 3,6%	—	113
20	Gmunden	20 ± 7,3%	30 ± 8,4%	—	27 ± 8,1%	30
21	Sachrang	—	64 ± 9,6%	64 ± 9,6%	—	25
22	Kufstein		+	+		9
23	Lienz	—	36 ± 7,7%	3 ± 2,6%	—	39
24	Wernberg	26 ± 5,2%	—	—	33 ± 5,6%	70
25	Raiblersee	1 ± 1,2%	57 ± 5,9%	—	—	70
26	Ferlach	—	13 ± 5,4%	—	—	39
27	St. Paul	—	—	—	—	36
28	Judenburg	41 ± 12,0%	—	29 ± 11,0%	—	17
29	Gleichenberg . . .	10 ± 3,9%	—	—	—	60
30	Dornbirn	—	33 ± 11,0%	—	—	18
31	Heiligenberg . . .		++	+		6
32	Gatersleben . . .	+				4
33	Berlin	38 ± 9,0%	—	—	—	29
34	Naumburg	26 ± 6,7%	2 ± 2,1%	56 ± 7,5%	—	43
35	Jena	2 ± 2,1%	10 ± 4,6%	47 ± 7,6%	10 ± 4,6%[1]	43
36	Würzburg	—	—	—	23 ± 11,5%	13
37	Castell	—	35 ± 6,6%	8 ± 3,8%	21 ± 5,6%	52
38	Reutlingen I . . .	—	30 ± 6,2%	16 ± 5,0%	6 ± 3,2%	54
39	Reutlingen II . .	—	35 ± 8,5%	23 ± 7,6%	—	31
40	Freiburg i. Br. . .	5 ± 5,0%	63 ± 11,0%	—	—	19

[1] Hier liegt der auf der Basis von VSa gebildete Strukturtyp V Je/St vor.

Inversionen keinen spezifischen Anpassungswert an bestimmte ökologische Bedingungen des Standortes haben können. So extreme Standorte wie die hochgelegenen Fundorte 22 (Kufstein), 23 (Lienz), 25 (Raiblersee) und 28 (Judenburg) zeigen keinerlei gemeinsame Eigentümlichkeiten. Die Verbreitungsgebiete der einzelnen Inversionen folgen keineswegs irgendwelchen ökologisch abgrenzbaren Bereichen, sondern sind offenbar rein geographisch bedingt. Die Nachprüfung des Standortes Hollenburg (Nr. 1: Werte von 1950, Nr. 12: Werte von 1952) nach 2 Jahren bestätigt die Feststellung, daß in so kurzen Zeiträumen

keine wesentliche Verschiebung in der Verteilung der Inversionstypen an einem Standort erfolgt (auch die Abweichung in der Häufigkeit der Inversion III Ku ist nicht statistisch gesichert). Unterschiede zwischen nahe benachbarten Standorten, die MAINX (l. c.) auf die relative Isolation der Bestände der Wirtspflanze zurückführt, lassen sich an weiteren Beispielen aufzeigen. Der Standort 18 (Gießhübl) liegt im Wiener Wald ungefähr zwischen den Standorten 2 (Kugelwiese) und 3 (Josefshöhe) einerseits und 4 (Gaaden) andererseits. Er zeigt gegenüber den Standorten 2 und 3 eine starke Abnahme der Inversion I Ku, die in 4 ganz fehlt, während für die in 4 ebenfalls fehlende Inversion III Ku ein solches Gefälle nicht nachweisbar ist. Die Inversion II Ku ist allen 4 Standorten gemeinsam. Besonders auffallend sind die starken Unterschiede in der Zusammensetzung des chromosomalen Polymorphismus zwischen den Standorten 24 (Wernberg) und 25 (Raiblersee), die in der Luftlinie nur 35 km voneinander entfernt sind. Zwischen ihnen liegen allerdings das Drau-, bzw. Gailtal, ein Zug höherer Bergrücken und das Kanaltal als isolierende Schranken. Es bestätigt sich an diesen Beispielen die Annahme, daß starke Unterschiede zwischen näher benachbarten Standorten auf Isolation im Vorkommen der Wirtspflanze zurückzuführen sind.

Die geographische Verbreitung der verschiedenen Inversionstypen ist in der Karte (Abb. 1) übersichtlich dargestellt, wobei die Verbreitungsareale der einzelnen Strukturtypen durch Linien eingegrenzt sind, soweit sich eine solche Abgrenzung aus den gewonnenen Stichproben durchführen ließ. Im großen und ganzen entspricht das Bild der Annahme von MAINX (l. c.), daß jeder Strukturtyp nur einmal an einer Stelle entstanden ist und sich dann durch die Population allmählich ausgebreitet hat und daß dementsprechend im Zentrum der Verbreitungsgebiete die Heterozygotenfrequenz am höchsten, an der Peripherie aber am geringsten ist.

Die Inversion I Ku scheint vom Norden in den östlichen Teil des Untersuchungsgebietes eingedrungen zu sein und sich vor allem entlang dem Donautal und in einem Querdurchbruch durch die Tauern ausgebreitet zu haben. Die höchste Heterozygotenfrequenz finden wir in 33 (Berlin), dann im Donautal an den Standorten 13, 1 (12), 11 und 3, ferner in 19 (Leithagebirge) und 28 (Judenburg). Gegen die Grenzen des Verbreitungsgebietes zu sehen wir einen deutlichen Abfall der Heterozygotenfrequenz von 34 (Naumburg) mit 26% gegen 35 (Jena) mit 2% und von 24 (Wernberg) mit 26% gegen 25 (Raiblersee) mit 1%, sowie in 29 (Gleichenberg) mit 10% und in 17 (Falkenstein) mit 16%. Die Inversion fehlt im gesamten westlichen Teil, ferner — mit Ausnahme von 2 Einbrüchen — im südlichen und südöstlichen Teil des Untersuchungsgebietes. Allerdings liegt eine Ausnahme vor, die einzige in

unserem gesamten Untersuchungsmaterial: Die Inversion I Ku wurde auch in dem zahlenmäßig nicht großen Material aus Freiburg i. Br. (40) gefunden, allerdings nur in einem Präparat. Vielleicht kann man hier

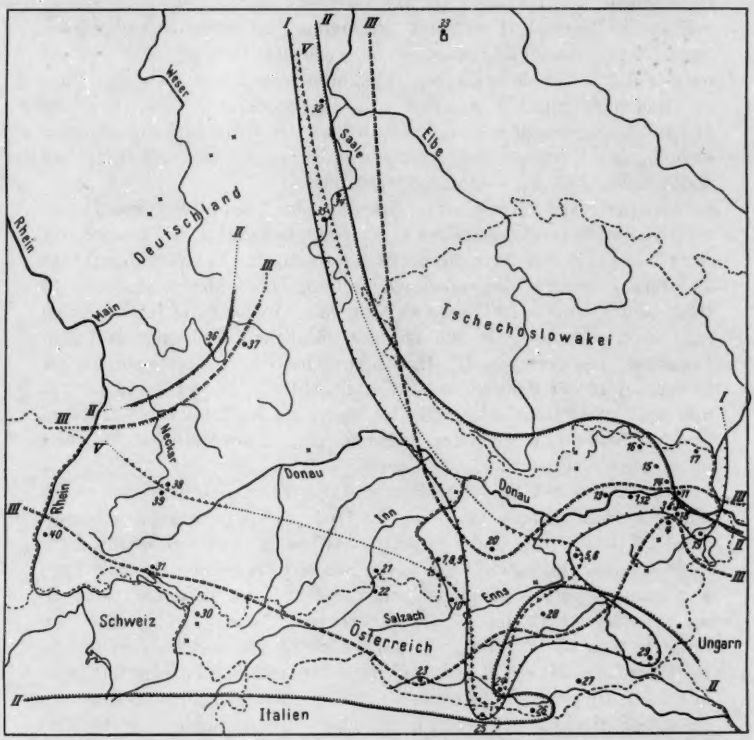

Abb. 1. Verbreitungsskizze der Inversionstypen I Ku, II Ku, III Ku und V Sa. Die Standorte sind mit ● und den in der Tabelle angeführten Nummern bezeichnet. Parasitenfreie Funde von *Lilium martagon* sind mit × bezeichnet. Die Verbreitungsgebiete der 4 Inversionstypen sind durch verschieden markierte Linien eingegrenzt, deren Marken gegen das Innere der Verbreitungsgebiete weisen

an eine Einschleppung durch den Menschen denken, etwa in Form von Importen von Holz oder Baumschulmaterial.

Die Inversion II Ku scheint sich vom Westen her ausgebreitet zu haben. Sie stößt einerseits gegen den mittleren Nordteil des Gebietes vor, andererseits zeigt sie eine erfolgreiche Ausbreitung durch die nördlichen Kalkalpen und entlang dem Donautal und einen weiteren

Vorstoß entlang den Karawanken. Sie ist mit hoher Heterozygotenfrequenz in 40 (Freiburg i. Br.), in 7—9 (Salzburg) und 10 (Werfen), ferner am rechten Donauufer (1 und 12, Hollenburg), in 2, 3 und 4 (Wienerwald), und in 5—6 (Lunz) vertreten, aber auch in 23 (Lienz) und 25 (Raiblersee). Der Abfall der Heterozygotenfrequenz gegen die Verbreitungsgrenzen zeigt sich von 35 (Jena) mit 10% gegen 34 (Naumburg) mit 2%, ferner am linken Donauufer mit 12% in 13 (Spitz) und 16 (Nieder-Fladnitz) und mit 4% in 14 (Stockerau). Die Inversion II Ku fehlt im Nordosten und Nordwesten des Untersuchungsgebietes, auch im nordöstlichen Teil von Niederösterreich, sie fehlt ferner im südöstlichen Teil des Untersuchungsgebietes.

Die Inversion III Ku zeigt eine ähnliche Verbreitung wie II Ku, doch ist ihr Verbreitungsgebiet stärker eingeschränkt. Sie besetzt mit der höchsten Heterozygotiefrequenz die Standorte 34 (Naumburg) und 35 (Jena), erreicht aber auch am rechten Donauufer in 1, bzw. 12 (Hollenburg) eine hohe Frequenz. In ihrer Ausbreitung in Österreich folgt sie wieder deutlich dem Zug der nördlichen Kalkalpen und dem Donautal. Die geringere Häufigkeit an den Grenzen des Verbreitungsgebietes zeigt sich deutlich in 37 (Castell) mit 8%, in 23 (Lienz) mit 3% und am linken Donauufer. Die Inversion III Ku fehlt im Südwesten und Nordwesten des Gebietes, ferner im ganzen nordöstlichen Teil und im Süden des Untersuchungsgebietes.

Die Inversion V Sa zeigt einen von den übrigen Inversionen etwas abweichenden Verteilungsmodus. In Österreich ist sie an den Standorten 25 (Raiblersee) und 20 (Gmunden) häufig, während sie mit 10% in 5—6 (Lunz) und mit 2% in 7—9 (Salzburg) gegen die Grenzen ihres Verbreitungsgebietes abzufallen scheint. Häufig ist sie dann wieder an den Standorten 36 und 37 (Würzburg und Castell), während sie im Südwesten in 38 (Reutlingen I) nur mehr mit 6% vertreten ist und in 39 (Reutlingen II) schon fehlt und nach Osten zu in 35 (Jena) nur mehr mit 10% Heterozygotenfrequenz auftritt, hier allerdings in dem abgeleiteten Strukturtyp V Je. Es ist nicht zu entscheiden, ob die Verbreitungsgebiete des Inversionstyps V Sa in Österreich und in Franken miteinander zusammenhängen. Aus den Gebieten, die hier eine Brücke schlagen könnten, konnte leider kein mit Parasiten befallenes Material gewonnen werden. In diesen Gebieten ist *Lilium martagon* nicht allgemein verbreitet und seine Bestände sind offenbar ziemlich isoliert. Es wäre möglich, daß ein ursprünglich geschlossenes Verbreitungsgebiet des Inversionstyps V Sa durch den Rückgang der Wirtspflanze in diesen Gebieten sekundär unterbrochen worden ist.

Wenn man die Verbreitungsgebiete der 4 verschiedenen häufigen Inversionen vergleichend betrachtet, so fällt es auf, daß bei den Inversionstypen II Ku und III Ku eine ausgesprochene Tendenz zur West-

Ost-Verbreitung entlang dem Zuge der Alpen, bzw. dem Verlauf des Donautales vorherrscht. Auch in der Ausbreitung von I Ku scheint das Donautal mit bestimmend zu sein. Die von MAINX (l. c.) angenommene radiäre Ausbreitung einer einmal entstandenen Inversion durch die Population ist offenbar durch die Verbreitungsart der Wirtspflanze, besonders durch den mehr oder weniger dichten Zusammenhang ihrer Bestände modifiziert. Geographische Barrieren, die die Geschlossenheit dieser Bestände unterbrechen, wirken auch für die Ausbreitung der Inversionstypen erschwerend. In Gegenden, in denen die Wirtspflanze nur in kleineren, mehr oder weniger isolierten Beständen vorkommt, können die Inversionstypen sich nur langsam ausbreiten. Wo aber die Wirtspflanze häufig ist und ihre Bestände in gutem Zusammenhang miteinander stehen, wie dies in den nördlichen Kalkalpen und in den das Donautal begleitenden Hügelketten der Fall zu sein scheint, ist die Ausbreitung der Inversionstypen erleichtert. Die Imagines von *Liriomyza* sind nicht sehr flugtüchtig und scheinen keine Tendenz zu Migrationsbewegungen zu haben. Sie befallen wahrscheinlich unmittelbar diejenigen Bestände ihrer Wirtspflanze, in denen schon ihre Eltern gelebt haben. Dies ist wohl die Voraussetzung dafür, daß sich die Verbreitungsgebiete der verschiedenen Inversionstypen und das Gefälle ihrer Häufigkeit bei diesem Objekt so rein abzeichnen. Der Verbreitungsmodus der Inversionstypen, bzw. die Abweichungen von einer rein radiären Verbreitung sind offenbar eine Funktion der Populationsdichte und des inneren Zusammenhanges der Population als Fortpflanzungsgemeinschaft und diese wieder ein Abbild der Populationsdichte der Wirtspflanze, an die *Liriomyza* fixiert ist.

Außerhalb der Verbreitungsgebiete der verschiedenen Inversionstypen ist stets nur die Standardanordnung des betreffenden Chromosoms homozygot vorhanden. Dies wurde für das I-Chromosom für die Standorte 7—9 (Salzburg), 30 (Dornbirn), 27 (St. Paul), 5—6 (Lunz) und 4 (Gaaden) durch Untersuchung bestätigt, ebenso für das II-Chromosom für die Standorte 33 (Berlin), 17 (Falkenstein), 27 (St. Paul), 29 (Gleichenberg), 28 (Judenburg) und 36 (Würzburg), für das III-Chromosom für die Standorte 33 (Berlin), 20 (Gmunden), 17 (Falkenstein), 4 (Gaaden), 27 (St. Paul), 24 (Wernberg), 30 (Dornbirn), 40 (Freiburg i. Br.) und 36 (Würzburg), für das V-Chromosom für die Standorte 33 (Berlin), 34 (Naumburg), 4 (Gaaden), 27 (St. Paul), 25 (Raiblersee), 30 (Dornbirn) und 40 (Freiburg i. Br.).

Die Inversionstypen I Ku, II Ku und III Ku erreichen in den Gebieten ihrer größten Häufigkeit in den Larven vielfach eine Heterozygotenfrequenz von 50%, ohne diese jedoch statistisch gesichert zu überschreiten. Die Inversion V Sa erreicht nur eine Frequenz von etwa 33% Heterozygoten. Dies weist darauf hin, daß die Ausbreitung der

Inversionstypen durch eine Heterosiswirkung erfolgt, die mit 50% Heterozygoten den höchsten Grad ihrer Auswirkung erreicht. Die statistische Verteilung der heterozygoten und der beiden homozygoten Konfigurationen wurde für die Inversionen I Ku, II Ku und III Ku an den Stellen ihrer größten Häufigkeit geprüft und entspricht innerhalb der Fehlergrenzen der Hardy-Weinberg-Formel. Eine selektive Bevorzugung der Heterozygoten dürfte wohl erst während der Metamorphose oder in der Imago erfolgen oder in physiologischen Eigentümlichkeiten der Imago bestehen, die sich nicht in einer Störung des Gleichgewichts nach Hardy-Weinberg äußern. Wo mehrere Inversionstypen an einem Standort vertreten sind, besteht zwischen ihren verschiedenen heterozygoten Zuständen keine Korrelation, wie mehrere Stichproben zeigten. Die durchschnittliche Inversionsdichte ist am höchsten am Standort 1, bzw. 12 (Hollenburg) und beträgt dort 1,4 Inversionen je Larve, im Wiener Wald zeigt sie Werte von 1,1 am Standort 3, von 0,6 an den Standorten 2 und 18 und von 0,5 am Standort 4. Eine Inversionsdichte von 0,6, bzw. 0,5 ist überhaupt ziemlich häufig, so an den Standorten 24 (Wernberg), 25 (Raiblersee), 37 (Castell) und 38 (Reutlingen I), obwohl die strukturellen Grundlagen der Inversionsdichte an diesen Standorten sehr verschieden sind. Am Standort 29 (Gleichenberg), wo nur ein Inversionstyp spärlich vorhanden ist, sinkt die Inversionsdichte auf 0,1 und der Standort 27 (St. Paul) ist der einzige des ganzen Untersuchungsgebietes, der frei von Inversionen ist.

Obwohl bei *Liriomyza* keine chromosomalen Unterschiede zwischen den beiden Geschlechtern festzustellen sind, ist es anzunehmen, daß ein Chromosom als genetisches Y-Chromosom wirksam ist. Da für die Inversionstypen I Ku, II Ku, III Ku und V Sa (und damit auch für den abgeleiteten Typ V Je) auch der homozygote Zustand der betreffenden Inversion festgestellt werden konnte, kann keine von diesen Inversionen im genetischen Y-Chromosom liegen, wie dies bei gewissen Inversionen von *Chironomus tentans* der Fall ist (Beermann 1955). Man könnte vermuten, daß die Inversion V Sa im genetischen X-Chromosom liegt, da diese Inversion als einzige nirgends die Heterozygotenfrequenz von 50% erreicht. Die höchsten Frequenzen dieser Inversion würden gerade ungefähr der Hälfte aller Weibchen entsprechen, also einem maximalen Heterosiseffekt im weiblichen Geschlecht. Es kann das Geschlechtschromosomenpaar allerdings auch durch das IV-Chromosom repräsentiert sein, das — bis auf einen einmaligen Befund (Mainx l. c.) — keine Inversionen zeigt.

Die Bedeutung des chromosomalen Polymorphismus in natürlichen Populationen wurde in den letzten Jahren an verschiedenen Objekten wiederholt statistisch und experimentell untersucht und die Problemlage wurde mehrfach übersichtlich dargestellt (z. B. Da Cunha 1955,

MAINX 1955). Verglichen mit anderen gut untersuchten Fällen stellt die Situation bei *Liriomyza urophorina* insofern einen Sonderfall dar, als hier der Einbau von durch Inversionen ausgezeichneten Strukturtypen in die Population erst in den Anfängen steht. Die einzelnen Inversionstypen haben eine relativ enge, geographisch gut abgrenzbare Verbreitung. Das Häufigkeitsgefälle innerhalb ihrer Verbreitungsgebiete weist auf ihre einmalige Entstehung hin, macht die Heterosiswirkung der strukturell heterozygoten Zustände als treibende Kraft für die Verbreitung der Inversionstypen wahrscheinlich und zeigt den Einfluß der Populationsstruktur der Wirtspflanze und damit auch des Parasiten auf diese Ausbreitungsvorgänge. Die außerordentlich stabilen populationsstatistischen Eigenschaften von *Liriomyza* und ihre Standortstreue lassen dieses Bild viel klarer hervortreten, als dies bei einem anderen Organismus überhaupt zu erwarten wäre.

Auch über das vermutliche phylogenetische Alter des chromosomalen Polymorphismus läßt sich bei *Liriomyza* einiges aussagen. Es wird allgemein angenommen, daß *Lilium martagon* während der Eiszeit in Europa eingewandert ist, wahrscheinlich aus dem nördlichen Asien. Es ist nicht zu entscheiden, ob der Parasit *Liriomyza urophorina* bereits mit seiner Wirtspflanze Europa besiedelt hat. Die Tatsache, daß er in der nordwestlichen Grenzzone des mitteleuropäischen Verbreitungsgebietes von *Lilium martagon* fehlt und daß er fast immer dort fehlt, wo die Bestände von *Lilium martagon* schütter sind oder Isolate darstellen, scheint darauf hinzuweisen, daß der Parasit erst später die mitteleuropäischen Bestände seiner Wirtspflanze befallen hat. Auch die Beobachtung, daß er in den hohen Lagen, die von *Lilium martagon* erst mit dem Rückgang der Gletscher besiedelt worden sind, meist fehlt, ließe sich in dem Sinne interpretieren, daß der Parasit in die europäischen Bestände erst eingedrungen ist, als seine Wirtspflanze ihre heutige Verbreitung bereits erreicht hatte. Ob man nun annimmt, daß der Parasit mit seiner Wirtspflanze in Europa eingedrungen ist oder erst später ihr gefolgt ist, auf jeden Fall muß die Verbreitung von *Liriomyza* bereits ihren jetzigen Stand erreicht haben, als sich der chromosomale Polymorphismus zu entwickeln begann. Wenn die Population von *Liriomyza*, die mit ihrer Wirtspflanze oder später nach Europa gekommen ist, bereits chromosomal polymorph gewesen wäre, könnten die Verbreitungsgebiete der Inversionstypen und deren Häufigkeitsgefälle nicht jenen regelhaften Aufbau zeigen, wie er oben geschildert wurde. Dieser zeigt, daß die Abweichungen von der für eine homogene Population theoretisch anzunehmenden radiären Ausbreitung der Inversionstypen vom Ort ihrer Entstehung aus nur auf Grund der gegenwärtigen Struktur der Population von *Lilium martagon* zu erklären sind. Die heutige Ausbreitung von *Lilium martagon* ist aber erst mit

dem Rückgang der Vergletscherung am Ende der letzten Eiszeit eingetreten. Wenn die Population von *Liriomyza* schon während der letzten Eiszeit chromosomal polymorph gewesen wäre, müßten sich die Gebiete der Vereisung, vor allem also das Alpenmassiv, als isolierende Schranken für die Ausbreitung der chromosomalen Strukturtypen noch heute deutlich abzeichnen. Dies ist aber nirgends der Fall. Man kann daraus wohl schließen, daß der Vorgang der Ausbreitung von durch Inversionen ausgezeichneten chromosomalen Strukturtypen bei *Liriomyza* nicht älter, wahrscheinlich aber jünger als 10000 Jahre, d.h. ebenso viele Generationen ist. Im Gegensatz zu den Fällen von hochgradigem chromosomalen Polymorphismus bei *Drosophila*-Arten der *obscura*-Gruppe und bei gewissen *Chironomus*-Arten stellt der chromosomale Polymorphismus von *Liriomyza* eine phylogenetisch relativ junge Erwerbung dar. Dem entspricht auch der primitive Zustand des Verteilungsbildes der Inversionstypen, das durch die stabilen populationsstatistischen Eigenschaften dieses Organismus in so reiner Form konserviert erscheint. In diesen Beziehungen nimmt *Liriomyza* eine beachtenswerte Sonderstellung unter allen bisher bekanntgewordenen Fällen ein.

Die Autoren möchten all den vielen Herren danken, die durch ihre Hilfe bei der oft schwierigen Beschaffung des lebenden Untersuchungsmaterials diese Arbeit ermöglicht haben. Sie sind bestrebt, die Untersuchung auf weitere geographische Gebiete auszudehnen, sofern dies möglich ist.

Zusammenfassung

1. Durch die cytologische Untersuchung der in *Lilium martagon* parasitierenden Larven der Agromyzide *Liriomyza urophorina* von 40 verschiedenen Standorten in Österreich und Deutschland wurde die Verbreitung der in 4 verschiedenen Chromosomen vorkommenden Inversionstypen festgestellt.

2. Die 4 Inversionstypen haben wohl abgegrenzte Verbreitungsgebiete, die einander zum Teil überschneiden. Es besteht kein Zusammenhang zwischen dem Vorkommen der Inversionstypen und ökologischen Eigentümlichkeiten des Standortes. In den zentralen Bereichen der Verbreitungsgebiete erreicht die Häufigkeit von 3 Inversionstypen je eine Heterozygotenfrequenz von 50%, die des 4. eine von 33%, gegen die Grenzen der Verbreitungsgebiete fällt die Heterozygotenfrequenz allmählich ab. Außerhalb der Verbreitungsgebiete der Inversionstypen ist stets nur die Standardanordnung der Chromosomen vorhanden. Die Inversionsdichte beträgt meist 0,5—0,6 Inversionen, stellenweise bis zu 1,4 Inversionen je Larve. Nur ein Standort war ganz frei von Inversionen. Es besteht keine Korrelation zwischen den verschiedenen Inversionstypen. In den Larven steht der heterozygote

Zustand mit den beiden homozygoten Zuständen im Gleichgewicht nach Hardy-Weinberg.

3. Es ist anzunehmen, daß jeder der 4 Inversionstypen an einem Punkt erstmalig entstanden ist und sich durch eine Heterosiswirkung seines heterozygoten Zustandes durch die Population ausgebreitet hat, wobei die Struktur der Bestände von *Lilium martagon* die Abweichungen von einer rein radiären Ausbreitung bedingt hat.

4. Verschiedene Argumente sprechen dafür, daß der chromosomale Polymorphismus von *Liriomyza urophorina* phylogenetisch nicht älter als 10000 Generationen ist, wahrscheinlich aber jünger. Er stellt einen besonders ursprünglichen Typus unter den bisher bekanntgewordenen Fällen von strukturellem Polymorphismus dar.

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Chromosoma, Bd. 8, S. 30—52 (1956)

From the Applied Fisheries Laboratory, University of Washington, Seattle

ELECTRON-MICROSCOPE STUDIES OF DROSOPHILA SALIVARY-GLAND CHROMOSOMES*

By

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With 25 figures in the text

(Eingegangen am 14. Oktober 1955)

Introduction

In conjunction with a series of experiments concerning the effects of X-rays on the embryological development of salmonoids, a preliminary study of chromosome sensitivity to x-radiation in this group was made. Doses as small as 25 r were found to cause sticking of chromosomes at anaphase in 200—400-cell embryos of the rainbow trout (*Salmo gairdnerii*) with intensified effects at higher doses.

In order to study these abnormalities in greater detail an electron-microscope study of trout embryos was initiated. The early results were not encouraging, probably as a result of the use of inappropriate fixatives, and the studies with this species were postponed until suitable fixatives and techniques could be found.

To test the effects of different fixatives on the structure of chromosomes as seen in the electron microscope it was desirable (1) to use cells in which every nucleus contained visible chromosomes when viewed with the light microscope, and (2) to select cells containing chromosomes which had been previously studied extensively with the light microscope. Salivary gland cells of *Drosophila melanogaster* fulfilled these requirements.

Materials and Methods

Drosophila melanogaster larvae approximately 2 mm in length were dissected in the fixative. The salivary glands were removed from the larvae, separated from the attached fat bodies and transferred to fresh fixative. The following solutions were used:

(1) 1% osmium tetroxide in Michaelis' veronal acetate buffer at p_H 7.4 (Palade 1952).

(2) Modified Zenker's fixative [1].

(3) 0.1% and 1.0% formaldehyde in Michaelis' veronal-acetate buffer at p_H 7.4.

The tissue was left in the fixative for varying periods of time from 20 to 120 minutes.

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[1] Fifteen cc H_2O, 5 gms. $HgCl_2$, 5 cc of 10% chromic acid, 0.2 gm Na_2SO_4.

The salivary glands were then removed with a pipette and washed with three changes of buffer, placed in 70% alcohol for 20 minutes, transferred to two changes in 95% 20 minutes each, and three changes in absolute ethanol for the same length of time, followed by treatment with equal parts of absolute alcohol and methacrylate monomer for one hour. The glands were then transferred through three 20 minute changes of 100% methacrylate monomer plus catalyst (Luperco CDB)[1]. A mixture of 80% N-butylmethacrylate and 20% methyl methacrylate was used, to which had been added two grams wet weight of catalyst per 100 cc of mixed monomer. The catalyzed monomer was placed in a glass tube and prepolymerized for approximately 20 minutes at 65° C then placed into No. 2 gelatin capsules. The salivary glands were transferred with a pipette to the top of the thickened plastic, then pushed to the bottom with a glass rod. Polymerization was completed in 12 hours at 47° C.

Some of the formalin-fixed samples were electron-stained with phosphomolybdic acid (0.001 M) in 70% alcohol. The staining was accomplished during the dehydrating procedure and after the material had been fixed.

After polymerization the end of the block containing the tissue was trimmed to the shape of a truncated pyramid with an exposed square for sectioning of approximately one-half mm per side.

Sections were cut with an ultramicrotome built according to a design of QUINTON, LEUBA and BENNETT, modified from the instrument of PORTER and BLUM (1953). Glass knives were used (LATTA and HARTMAN 1950). The sections were floated on to 30% acetone in distilled water and picked up on formvar-coated Lektromesh[2] nickel grids, 150 meshes per inch. Thickness of the sections was determined by light interference colors (PORTER and BLUM, 1953) and those less than 500 Å thick were selected. Sections used for high resolution photographs were 300 Å thick or less. Methacrylate was not removed from the sections prior to viewing.

The grids were examined in an RCA-EMU-2C microscope, afforded by Dr. H. S. BENNETT. The microscope was equipped with a biased gun, an objective aperture about 25 μ in diameter, and a condenser aperture 250 μ in diameter. For fine focusing, a wide-field binocular microscope at a magnification of 9 × was used in conjunction with a superfine focusing control added to the microscope at the University of Washington.

Photographs were taken at magnifications of 3700 to 10,500 times and magnified further by photographic enlargement. Dow Latex 580-G was photographed for calibration.

Kodak Lantern Slide Medium plates were used and developed five minutes at 20° C in Kodak D-23. Enlargement prints were made on Kodabromide paper.

Results

1. Light microscopy

The effects of different fixatives upon the dimensions and optically visible structure of salivary chromosomes were studied by means of phase-contrast microscopy. When a modified Zenker's fixative was used, marked shrinkage of the chromosomes occurred and they assumed an alveolar configuration, especially within the banded areas. Fixation with buffered osmium tetroxide caused almost no shrinkage, but resulted

[1] 50% 2,4-dichlorobenzoyl peroxide with dibutyl phthalate. Lucidol Division, Novadelagene Corporation, Buffalo, New York.

[2] C. O. Jelliff Manufacturing Corporation, South Port, Connecticut.

in a fine reticulation of the entire nucleus. When buffered formalin (0.1% or 1.0% formaldehyde) was used chromosomal shrinkage was not observed nor was there any marked alteration in the homogeneous appearance. These findings agree favorably with those of METZ (1935b) and DOYLE and METZ (1935). Excellent detailed descriptions of salivary chromosome reactions to different fixatives are found in their papers.

2. Electron microscopy

No difference in chromosome structure was observed in electron photomicrographs with the use of concentrations of 0.27% to 2.7%

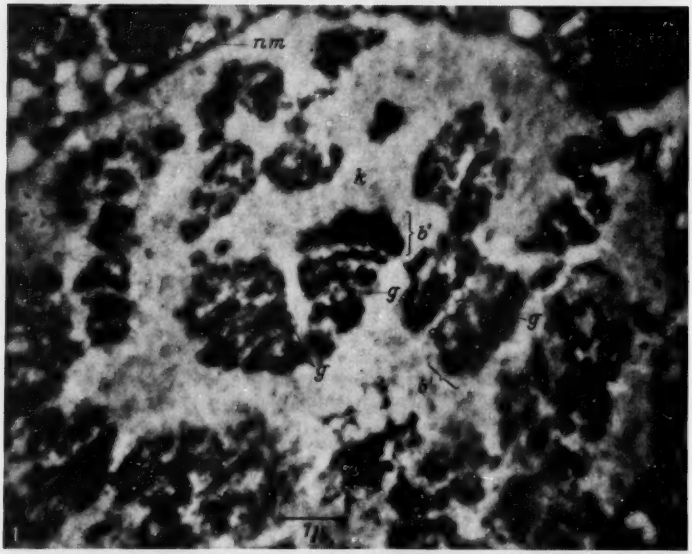


Fig. 1. An electron photomicrograph of part of a nucleus in a larval salivary gland cell of *Drosophila melanogaster*. Granules (*g*) are shown in the submicroscopic bands (*b*) and in the light microscopically visible bands (*b'*). There is no evidence of a chromosomal membrane in this or in any other of the photomicrographs although the nuclear membrane (*nm*) is present. The nucleolus (*n*) and the karyoplasm (*k*) are shown

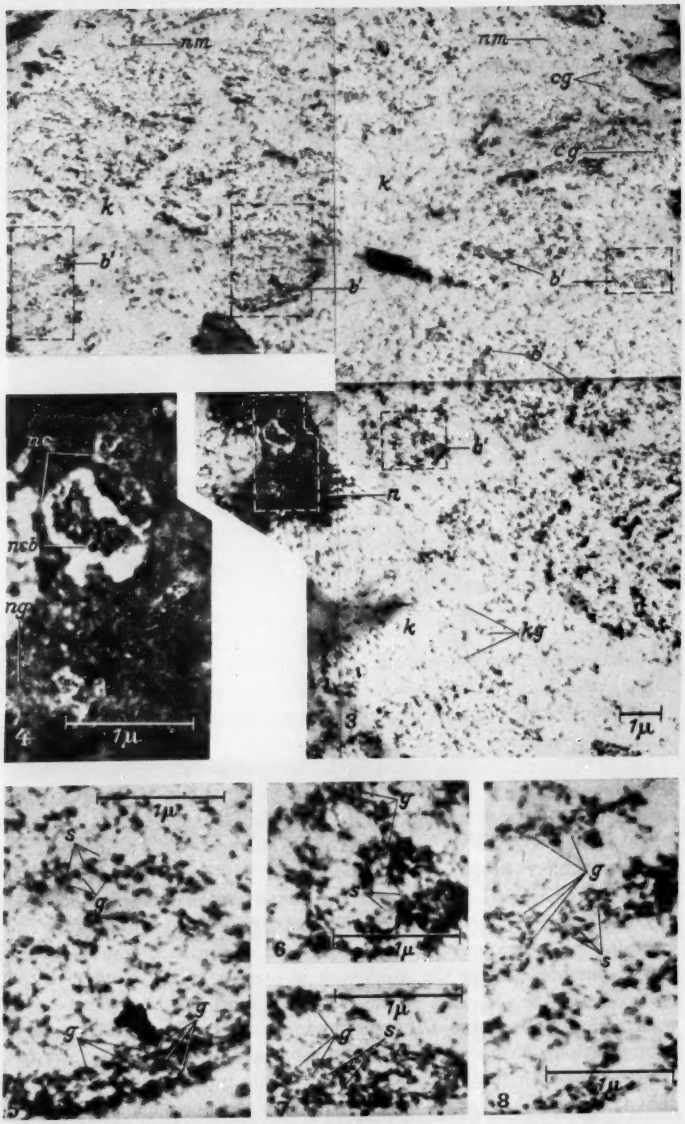
formalin. An advantage of formalin fixation is that it does not act as a stain as do the metallic salts used in some fixatives. Thus observed differences in density may more safely be attributed to varying densities of the components themselves or of their fixation products rather than to attached metal ions such as osmium. There is, of course, an attendant disadvantage with this method. Fine protein fibrils and globular proteins

within the salivary chromosomes are approximately the same density as that of the surrounding material, and thus are difficult to detect in the photomicrographs.

Nucleoprotein, however, exhibits great electron scattering power even in unstained animal cells. In spermatozoa of the silver salmon *(Oncorhynchus kisutch)*, sections 200 Å to 300 Å thick show only the nucleoprotein of the spermatozoan head to be very dense (unpublished observation). Other cells show similar results in regions of known high nucleoprotein concentration although to a lesser degree than those found in spermatozoa. In formalin-fixed sections of salivary gland chromosomes 500 Å or more thick the chromosome bands are so opaque that little detail can be observed although there often are indications that the band areas are composed of granules (*g*) (Fig. 1). Between the light microscopically visible bands are found, even in the thicker sections, submicroscopic bands of less than 1000 Å in width (Figs. 1 and 2). In some instances the wider bands appear to be formed by several closely apposed submicroscopic bands (Fig. 2). In thinner sections fine strands connecting successive bands are sometimes visible (Figs. 5, 6, 7 and 8). The diameter of the strands cannot be accurately determined in unstained formalin-fixed material but in stained chromosomes it is approximately 100 Å.

Fig. 2. An electron photomicrograph showing six submicroscopic bands (*b*) which occupy approximately six-tenths micron of linear chromosome length. The granular nature of the bands is shown (*g*). The light microscopically visible band (*b'*) appears to be composed of several closely apposed submicroscopic bands. One-tenth percent formalin in Michaelis' veronal-acetate buffer (p_H 7.4) with two hour fixation was used in the material for Figs. 1 and 2

In thinner sections an indication of the substructure within the light microscopically visible bands is found. In photomicrographs of such sections (Figs. 5, 6, 7 and 8) there are found, within the banded areas (b^1), many submicroscopic dense granules 200–300 Å in diameter. The granules are interconnected by a meshwork of fibrils of approximately 100 Å diameter. Since the meshwork is present in the band material regardless of the direction of the section, it must be three-dimensional within the chromosome although predominantly of a spirally wound configuration. Evidence that the structure of the band fibrils may not be identical with that of the interband fibrils is suggested by the fact that the band fibrils are visible in very thin sections whereas the inter-



Figs. 3—8

band fibrils usually are not. This may, of course, be due to the presence of additional protein, a thin layer of nucleic acid encrusted upon the fibrils within the band areas (COLE and SUTTON 1941), or both.

In addition to the granules found in the band areas, dense granules are found in the interband zones. HEITZ and BAUER (1933) reported a weak Feulgen reaction between the bands and COLE and SUTTON (1941), on the basis of U-V absorption studies, postulated the presence of some nucleic acid within these areas, possibly 10–20% that of the total protein content. SCHULTZ (1947) reported that there was probably nucleic acid in the interbands although he indicated that the amount was not great. MAZIA (1950), on the basis of enzyme studies, suggested that the nucleoprotein was concentrated in the bands but ran through the interbands in the form of strands. HERSKOWITZ (1952) described "a multitude of granules" in the interband areas in electron micrographs of acetic-acid-fixed and stretched *Drosophila* salivary chromosomes. HEILWEIL *et al.* (1952) reported numerous fine bands in the interband areas in electron photomicrographs of similarly fixed chromosomes. HEILWEIL described the finest bands as composed of transverse rows of granules approximately 300 Å in diameter. This configuration of the granules is confirmed in the present study of chromosomes prepared with Zenker's fixative.

Submicroscopic granules are also present within the karyoplasm of the nucleus. They differ from those of the band and interband areas of the chromosomes, however, in two ways. First, the individual granules in the karyoplasm are not as condensed as those of the chromosomes, and second, they are associated with thicker strands of material, probably protein. The diameter of the strands is approximately 200 Å.

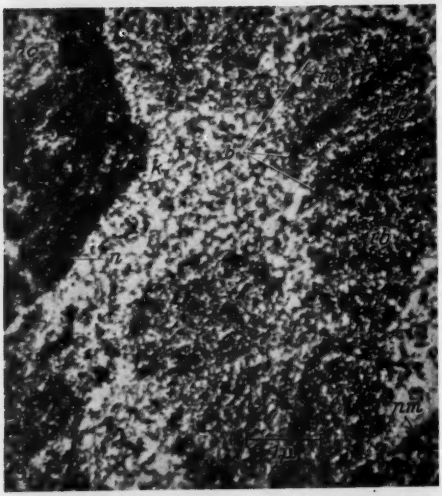
In addition to the granules in the karyoplasm, cytoplasmic granules are found surrounding the nuclear membrane in an area about 10,000–20,000 Å in width. The size of these particles approximates that reported by TSUBOI *et al.* (1954) for ribose nucleoprotein particles in the sea urchin egg, in which a minimum diameter of approximately 300 Å was reported, the assumption being that the particles are spherical.

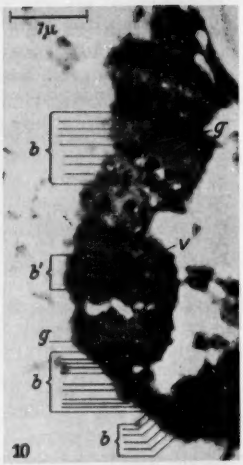
Because the interband fibrils could not be convincingly demonstrated in formalin-fixed material an attempt was made to stain them with an "electron stain". After formalin fixation the tissue was subjected to the action of phosphomolybdic acid. Gross artifacts were produced both

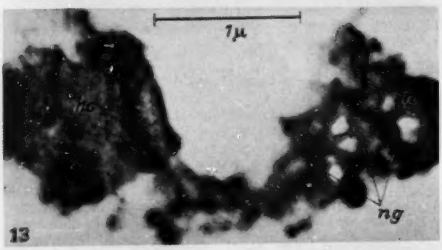
Fig. 3. An electron photomicrograph of a thin section which includes most of the nucleus of a larval salivary gland cell of *Drosophila melanogaster*. In the upper right and left corners of the photograph is the nuclear membrane (*nm*) outside of which is located cytoplasmic granules (*cg*), probably composed of ribonucleoprotein. Inside the nucleus are the nucleolus (*n*), karyoplasm (*k*), and the chromosomes. The light microscopically visible bands of the latter (b^1) are shown

Fig. 4. An enlarged inset of the nucleolar area in the photomicrograph in Fig. 3. The nucleolus is composed of electron dense granules (*ng*) 300 Å to 600 Å in diameter. Within the nucleolus are nucleolar cavities (*nc*) which are probably sections of canals which extend through the structure. Within the cavities is located material (*ncb*) similar in appearance to that of the euchromatic chromosome bands. One-tenth percent formalin in Michaelis' veronal acetate buffer (p_H 7.4) with two hour fixation was used in Figs. 3, 4, 5, 6, 7 and 8

Figs. 5, 6, 7 and 8. Enlarged insets of areas outlined in the photomicrograph in Fig. 3 are shown. Bands in the chromosomes are composed of granules (*g*) 200 Å to 300 Å in diameter interconnected by strands (*s*) approximately 100 Å in diameter. There is random directional orientation of the strands with some tendency for a configuration tangential to that of the chromosome axis. Karyoplasmic granules (*kg*) are present outside of the chromosomes







Figs. 9—14

in the chromosomes and nucleoplasm, but the presence of a greater concentration of protein in the interband areas than within the karyoplasm was demonstrated. The vesicular artifacts, probably induced by the acidic metal stain, destroyed the normal configuration of the material within both the band and interband material, although fine fibrils of approximately 100 Å diameter vere found in the latter (Fig. 9).

The results of fixation with buffered osmium tetroxide were similar to those described by BORYSKO (1953) and will not be discussed here. It was noted, however, that with osmium tetroxide fixation, the nucleic acid in the bands appeared to become more or less depolymerized with an attendant breakdown of visible organization. BAIRATI and LEHMANN (1954) have suggested that this may be caused by the inability of osmium tetroxide to precipitate structures rich in nucleoproteins fast enough, or completely enough to preserve their structure.

Salivary gland chromosomes fixed in modified Zenker's solution contain fixation artifacts similar to those described by other workers using acetic acid. In general, however, the vesicles or alveoli demonstrated in the present work are smaller than those found after fixation with acetic acid. Even the acid-fixed (Zenker's) material, however, is useful for studying the structure of salivary chromosomes, especially in view of the interpretations of the fixed structure previously proposed by METZ (1935a, b).

Fig. 9. A photomicrograph of a larval salivary gland cell of *Drosophila* fixed for two hours in 5 % formalin in Michaelis' veronal-acetate buffer of pH 7.4, then stained with 0·001 M phosphomolybdic acid in 70 % alcohol for 1½ hours. A greater concentration of stained material (probably protein) is indicated in the interband areas (*ib*) of the chromosome than in the karyoplasm (*k*). The granular nature of the nucleolus (*n*) is indicated and within the nuclear cavities (*nc*) is located material similar to that found in the euchromatic chromosome

Figs. 10, 11, 12, 13 and 14. Tissues fixed with a modified Zenker's fixative (15 cc water, 5 gm mercury bichloride, 5 cc of 10 % chromic acid, and 0.2 gm sodium sulfate). Not buffered. Figs. 11 and 14, tissues were fixed forty minutes, the remaining tissues were fixed twenty minutes

Fig. 10. A photomicrograph showing the presence of many submicroscopic bands (*b*) formed of granules (*g*). In the light microscopically visible bands (b^1) a vesicular configuration (*v*) is shown. Marked chromosomal shrinkage and structural artifacts are produced when Zenker's fixative is used

Fig. 11. A photomicrograph of part of a salivary gland chromosome in which submicroscopic vesicles (*v*) have coalesced to form the walls of larger light microscopically visible vesicles 0.2 μ in diameter. The interband granules (*ig*) are not forced into a vesicular configuration

Fig. 12. An electron photomicrograph of a chromosome in which many small bands (*b*) formed by submicroscopic granules are shown. There are, on the average, ten submicroscopic bands per linear micron of euchromatic interband chromosome

Fig. 13. A photomicrograph of the nucleolus and heterochromatin in a larval salivary gland chromosome. The nucleoprotein granules of the heterochromatin (*hc*) are not forced into the vesicular configuration found in euchromatic chromosomes, but rather form many isolated aggregates of dense material. The nucleolus contains many dense granules (*ng*) approximately 200 Å in diameter in an amorphous matrix

Fig. 14. A photomicrograph of a salivary gland chromosome in which the band material (b^1) has been disrupted into a vesicular configuration. The vesicles (*v*) are 300 Å to 800 Å in diameter with walls approximately 200 Å thick

In chromosomes fixed with Zenker's solution the bands are disrupted into distinct vesicles, 300 Å to 800 Å in diameter (Figs. 10, 11 and 14) with walls approximately 200 Å thick. These vesicles in turn may coalesce to form large microscopic vesicles 2000 Å or more in diameter (Fig. 11). The walls of the submicroscopic vesicles contain the denser material of the bands. The material enclosed within the vesicles is more dense than the karyoplasm, though less so than the vesicle walls, and probably comprises the nonfibrous protein of the chromosomes.

In the interband areas the scattered granules of dense material are usually not forced into the vesicular or alveolar configuration found within the bands. This may be due to the relatively great distance between individual granules as compared with that of the band granules. Rows of homologous granules appear in the photomicrographs as submicroscopic bands within the interband areas (Figs. 10 and 12) and there are, on the average, ten submicroscopic bands per linear micron of interband material in *Drosophila* salivary chromosomes. The number of submicroscopic bands within the microscopically visible bands cannot be dtermined accurately because of their disorientation by the vesicles.

In electron photomicrographs of salivary chromosomes the heterochromatic nucleoprotein does not appear to be as well organized as that of the euchromatic bands. In the former, the nucleoprotein tends to agglomerate into small clumps within the chromosomal matrix. The apparently disorganized configuration of the chromosomes in the heterochromatic areas has precluded their positive identification in very thin formalin-fixed sections. In thicker sections the substructure of the nucleoprotein clumps is masked by loss of resolution.

Chromosomes fixed with modified Zenker's solution exhibit different reactions in the heterochromatic and euchromatic areas. Whereas euchromatic nucleoprotein assumes the form of submicroscopic vesicles which, in turn, form larger ones, the tendency for vesicle formation in heterochromatic nucleoprotein is greatly reduced (Fig. 13). If the vesicular walls in the euchromatic bands are formed by coagulation of fibrils this may well indicate a reduction of the fibrillar structure in the heterochromatic regions in the living condition.

In no preparation was an indication of a chromosomal membrane found. The significance of this observation is in the fact that, with all the techniques used, the nuclear membrane, plasma membrane, mitochondrial walls, and the endoplasmic reticulum were always present and identifiable.

The nucleolus, in formalin-fixed material, is composed of a central aggregate of ill-defined granules 300 Å to 600 Å in diameter. Within this structure there is a central cavity and, in addition, several smaller cavities in the surrounding nucleolar material. Within all of the cavities

is located material similar in appearance to that of the euchromatic bands. In Fig. 3, karyoplasmic nucleoprotein appears to radiate out from the nucleolus although this may be a fixation artifact.

In nucleoli subjected to Zenker's fixative the dense material of the nucleolar aggregate is condensed into well defined granules (*ng*) of approximately 200 Å diameter located in an amorphous matrix (Fig. 13).

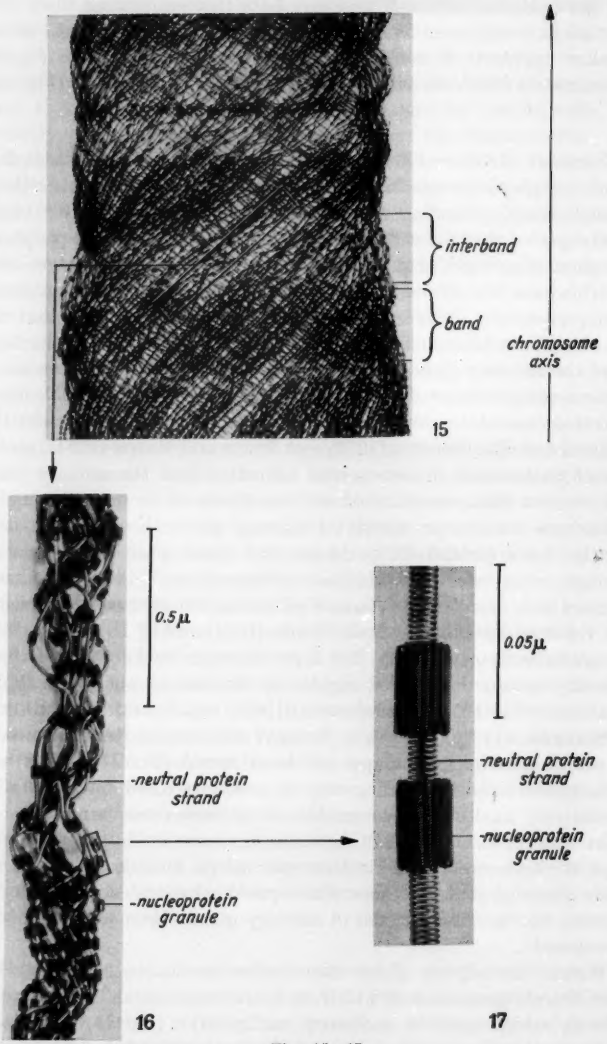
Discussion

KOLTZOFF (1934) and BRIDGES (1935) advanced the hypothesis that the salivary gland chromosomes were composed of sixteen visible strands (chromonemata) extending the length of the chromosome. KOLTZOFF (1934) reported that the strands followed a spiral course on the periphery throughout the length of the chromosome. BAUER (1935) observed that in *Chironomus* the strands followed a spiral course but were not distributed peripherally. METZ (1935a and b), however, calculated that the individual units (chromonemata) would be submicroscopic if the large size of the salivary gland chromosomes were caused by reduplication of chromonemata without division. In the same year MULLER calculated the average size of the chromonemata to be no greater than $^1/_{50}\,\mu$ (200 Å). KURNICK and HERSKOWITZ (1952) and SWIFT and RASCH (1954), on the basis of photometric measurements, estimated that the salivary gland chromosomes each consist of about one thousand strands.

Electron microscope studies of salivary gland chromosomes, until recently, have yielded little information about their fine structure although, even in acetic-acid-fixed preparations, indications of substructure have been found. A basic fibrillar unit 50–100 Å in diameter has been reported by BEERMANN and BAHR (1954) and by HOVANITZ (1953) and granules approximately 300 Å in diameter have been described, especially within the banded regions by BEERMANN and BAHR (1954), HEILWEIL *et al.* (1952), HERSKOWITZ (1952), and SCHULTZ, MACDUFFIE and ANDERSON (1949). However, not until chromosome fixatives suitable for use in electron microscopy had been found (BORYSKO 1953) and methods developed for making very thin sections could detailed studies of relatively unaltered fine structure of chromosomes be made.

In view of the agreement between the calculated size of salivary gland chromonemata and electron microscope findings and from the observations of other workers with optical, chemical and micrurgical methods, the following model of salivary gland chromosome structure is proposed.

Within the salivary gland chromosome are located a thousand or more strands (genonemata) which are more or less regularly wound into a loosely cabled spiral or corkscrew configuration (Fig. 15) (BEERMANN 1952). Each strand extends throughout the length of the chromosome


interband
band
chromosome axis
15
0.5 μ
neutral protein strand
nucleoprotein granule
16
0.05 μ
neutral protein strand
nucleoprotein granule
17

Figs. 15—17

in a spiral course but may, in some places within the chromosome, deviate from the general configuration.

At intervals — approximately 1000 Å in the interbands — each strand is covered by a granule 200–300 Å in diameter of desoxyribonucleoprotein (Figs. 16 and 17). In the banded areas the distance between granules is much less with the intervals ranging from 100–400 Å. The nucleoprotein is attached to the strand material which is not continuous through each nucleoprotein granule but, rather, connects successive nucleoprotein granules into a chain throughout the length of the euchromatic chromosome segments. The heterochromatic areas of the chromosome are very similar in construction except that the strand material is lacking or greatly reduced in amount. It is generally agreed that the strands are composed of protein. SERRA (1947) reported the "continuous skeleton" to be of nonbasic protein upon which the nucleoproteins were attached at certain points. MAZIA (1954) reported also that the backbone of the chromosome was protein. MIRSKY and RIS (1947) found that a tryptophane-containing protein comprised most of the residual protein after the histone and DNA had been removed. This they considered to be the material of the thread-like chromosomal strands.

Later work of MIRSKY and RIS (see MIRSKY 1951) indicated that the structural continuity of the chromosome is not dependent upon the basic protein portion because those proteins can be removed without altering the microscopically visible structure. The observations of MAZIA and JAEGER (1939), MAZIA (1941), KODANI (1942), CATCHESIDE and HOLMES (1947), and MCDONOUGH, ROWAN and MOHN (1952) suggested that the structural integrity of the chromosome is not dependent upon the presence of desoxyribonucleic acid. However, if chromosomes from which the histones have been removed are treated with DNAse the structure of the chromosome is destroyed and only small coiled fragments

Fig. 15. A diagram of a model of part of a larval *Drosophila* salivary gland chromosome is shown, including interbands and bands. The chromosome is composed of approximately one thousand genonemata (strands), each of which extends throughout the length of the chromosome. The genonemata follow a spiral course throughout the length of the chromosome. Banded areas are caused by a greater concentration of nucleoprotein granules along the strands in given areas

Fig. 16. A model of a few of the strands shown in Fig. 15. Each genonema is composed of nucleoprotein granules interconnected by strands of neutral protein. The space between adjacent granules in the banded areas is 100 Å to 300 Å and in the interbands 700 Å to 900 Å. There is also a globular protein portion of the chromosome which accounts for 80 to 95 % of the chromosome volume. The globular material is not indicated, but would fill the spaces between the strands

Fig. 17. A model of two of the nucleoprotein granules with the associated strand protein is shown in Fig. 15. The strand protein is probably composed of a protein α-helix coiled into a strand about 100 Å in diameter. The granules are formed of nucleoprotein molecules, approximately 20 Å by 6000 Å, folded into a tubular structure about 300 Å in length and having an outer diameter of 200 Å. The strand material is discontinuous but joins successive nucleoprotein granules by bridges of divalent cations (Ca, Mg or both)

of residual protein remain (MIRSKY 1951). Thus in the unaltered chromosome the short longitudinal strands of protein are probably joined into a continuous genonema by the nucleoprotein granules with both the basic protein and the nucleic acid attached to the strands. The protein of the strands is probably the "residual protein" of MIRSKY and RIS, the nonbasic protein of SERRA being the same material.

D'ANGELO (1946, 1950) found that isolated salivary gland chromosomes could be stretched to ten times their original length and, upon release, would return to their former size. In view of the work of PAULING and COREY (1951) (see also RUDALL 1953) this marked elasticity of salivary gland chromosomes cannot be explained entirely by the stretching of the polypeptide chain. The 50 Å to 100 Å strands found in electron photomicrographs of salivary gland chromosomes, therefore, are probably each composed of a single polypeptide helix of approximately 10 Å in diameter coiled into a larger helix of approximately 100 Å diameter (Fig. 17) the gyres of which are stabilized one to the other by the types of junctions suggested by FREY-WYSSLING (1953) for parallel polypeptide chains. Such a helix would be capable of elongation 12 to 20 times without disruption of its structural integrity. Additional elasticity could be accounted for by the alignment of the more or less regular cable of strands into a more precisely parallel configuration. That this may be the case is suggested by the birefringence studies of SCHMIDT (1939, 1941) and PFEIFFER (1941, 1952).

Further indication that a single polypeptide helix may comprise the genonema is found in the studies of LEA and CATCHESIDE (1942) with *Tradescantia*. On the basis of X-radiation experiments these authors estimated that approximately seventeen ionizations would be required to break an interphase chromatid of that species. An interphase chromatid approximately 100 Å in diameter composed of *closely packed* α-helices would contain between nineteen and eighty-five helices in cross section. More than seventeen ionizations would be required to break this type of cable unless each ionization were to produce a chain reaction involving more than one molecule as suggested by SPARROW, MOSES and DUBOW (1952). If, however, the genonema is composed of a single coiled α-helix, seventeen ionizations could break the helix and loosen the bonds between adjacent gyres in the area of the break, thus producing a chromosomal break.

The configuration of the nucleohistone complex and its structural relation to the strand material are of interest in both the study of chromosome and gene structure. That the nucleoprotein within the salivary gland chromosome is in the form of granules 200–300 Å in diameter rather than in an extended configuration is almost certain. The work of SCHMIDT (1941) and AMBROSE and GOPAL-AYENGAR (1952) with the polarization microscope indicates that the long chain nucleoprotein molecules are arranged parallel to the axis of the chromosome, a condition which could not exist were the nucleoprotein molecule coiled around the chromosome strands (SCHULTZ 1947). It is, therefore, probable

that folding of the nucleoprotein molecule occurs, a possibility advanced by MULLER (1947) as an explanation for gene specificity. The axes of the folded sections of the molecule would be parallel to that of the chromosome.

If a nucleoprotein molecule (approximately 20 Å by 600 Å when opened out) were lapped at 300 Å intervals to form a sheet-like structure, then folded into a tube with an inner diameter of 100 Å, a granule with a length of 300 Å and a diameter of approximately 150 Å would result. The major part of the axis of such a granule would be parallel to that of the chromosome strand (Fig. 17). However, the folded configurations of the different successive granules along the genonema would not necessarily be identical.

The number of nucleoprotein granules along the length of the interphase-type genonema in a salivary gland chromosome must be very large. On the basis of the electron microscope findings, the total number of granules that would be present in a diploid set of interphase chromosomes is in the tens of thousands. This is in agreement with the report of KURNICK and HERSKOWITZ (1952) that the haploid set of *Drosophila* chromosomes would contain 44,000 molecules of DNA (MW, 10^6) with a mean distance between successive molecules of approximately 170 Å.

In view of the great number of granules it is improbable that each, with its associated strand protein, could represent one gene, although there are probably more genes than had previously been supposed. This assumption is suggested by the work with *Neurospora* (BEADLE 1946 and 1948, and HOROWITZ and LEUPOLD 1951), and with *Drosophila* (LEWIS 1945 and 1950, and GREEN and GREEN 1949).

Although the nucleoprotein granules may not each represent a gene, they correspond approximately in size, at least, to the X-ray sensitive areas of the genes. Using the X-ray target method, BLACKWOOD (1931 and 1932) derived a target size of 200 Å diameter and MULLER (1940) less than 100 Å. Accepting the postulate of WATSON and CRICK (1953) that the DNA is in the form of intertwining helices with an average diameter of approximately 20 Å, estimates of the volume of the desoxyribonucleoprotein molecule vary between 8×10^5 and 2×10^6 (Å)^3 (DEROBERTIS 1954 and BERNSTEIN and MAZIA 1953). CRICK and WATSON, however, suggest that the volume may be greater. The volumes of the targets reported by MULLER and BLACKWOOD are approximately 5×10^5 and 4×10^6 (Å)^3 respectively. On the basis of these estimates, the target area would contain from 0·2 to 4 nucleoprotein molecules. SCHULTZ (1941 and 1947), MAZIA (1941) and MULLER (1941) reported that the nucleic acid of the chromosomes is present as low polymers.

The nucleoprotein may correspond to the radiation sensitive area of the gene, but may not necessarily represent the gene itself. It may be

that the nucleoprotein portion does not possess the specificity of the gene, but rather only the ability to produce the structure that does contain the specificity. The idea that the specificity may be contained within the strand material is suggested by both immunological studies (Mirsky 1951) and the findings concerning heterochromatin. Schultz (1947) reported that the structural difference between the chromocentral heterochromatic regions and the euchromatic areas exists in the material of the interband areas and probably involves the elastic protein (strands). He also supported the proposal that the chromatic material of the heterochromatin is composed of duplicate genes and that the strand material does not form between repeated genes. If the heterochromatic areas differ structurally from the euchromatic regions by the absence of strand material, then differences in genetic activity between the two types of chromatin might well be dependent upon the presence or absence of strand material.

Genes of the heterochromatic areas do not exhibit the specificity associated with euchromatic genes and according to Schultz the lack of specificity in heterochromatin implies that gene specificity is separable from the other gene characteristics. Although this is in agreement with the concept that gene specificity resides in the strand material, it does not entirely preclude the possibility that the desoxyribonucleoprotein granules may possess the specificity of the gene. The mechanism which renders the granules incapable of producing strand material might also block their synthesis of gene specific materials or their precursors.

If the nucleoprotein granules and strand material (residual protein) of salivary gland chromosomes are normal constituents of chromosome structure, then the salivary gland chromosomes represent prophase chromosomes tightly coiled at the first division (Fig. 23a) in which reduplication without separation has occurred as a consequence of the intertwining of the newly formed pair (Fig. 23b).

The diploid interphase chromosomes of *Drosophila* probably have the same lengths and diameters of the strands and granules as those found in the salivary gland chromosomes if one allows for the slight loss of length in the giant chromosomes which results from their lose coiling. The euchromatic interphase chromosome would, thus, consist of a continuous chain of nucleoprotein granules, each attached to and surrounding one end of a polypeptide strand previously formed by that granule, with the other end of the strand attached to the next granule in the chain.

On the basis of the electron-microscope findings in the present study, each unit (strand plus nucleoprotein) would be from 400 Å to 1200 Å in length and from 100 Å to 150 Å in diameter.

BERNSTEIN and MAZIA (1953) extracted nuclear desoxyribonucleoprotein with distilled water after previous treatment with dilute citrate. The extracted particles were approximately 4000 Å long and 200 Å wide. MAZIA (1954) reported that these particles represent macromolecular chromosome fragments and constitute the chromosome when held together in linear sequence by bridges of divalent cations (Ca, Mg, or both). The particles tend to join together at the ionic concentrations normally existing in the cell and repel each other at lower ionic concentrations.

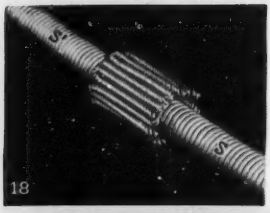
The present model of chromosome structure differs essentially from that of MAZIA only with respect to minimum particle size. The particles observed with the electron microscope in intact chromosomes, however, may comprise subunits of the particles of BERNSTEIN and MAZIA, and if so there are, in addition to the divalent bridges, other connections in the linear attachment of chromosomal subunits that are not affected by reduction of ionic strength.

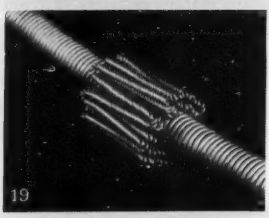
Assuming that the interphase chromosome is composed of granules separated by strands 200 Å to 1000 Å in length, reduplication might occur as follows:

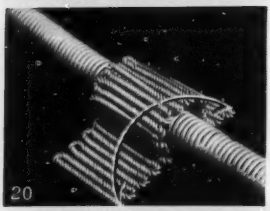
During telophase the euchromatic chromosomes commence despiralization and by late telophase or early interphase have despiraled completely to the 100-Å-strand condition. During interphase, nucleic acid is reduplicated in the manner postulated by WATSON and CRICK (1953) (see also DELBRÜCK 1954) at each granule so that a duplicate is formed with respect to both molecular succession and configurational folding (Figs. 18 and 19). The newly formed nucleic acid then synthesizes its histone-like protein in the image of the protein attached to the original granule. Upon completion of the reduplication process, the newly formed nucleoprotein unrolls from the original granule, the two edges of the sheet-like structure each rolling through 180° to form a new tubular nucleoprotein granule lying at the side of and parallel with the original (Figs. 20 and 21). The strand material is then synthesized within the column of each new granule parallel to the previously existing strand toward its neighboring granule where it becomes attached (Fig. 22). When the strand-to-granule attachments have occurred throughout the length of the genonema, reduplication is complete.

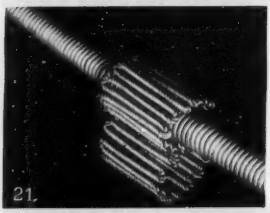
Evidence as to the time of reduplication of nucleoprotein during the normal mitotic cycle is contradictory. The work of PASTEELS and LISON (1950) and PELC and HOWARD (1952) indicated that reduplication takes place during the late telophase and early interphase. Histophotometric data of other authors (SWIFT 1950; POLLISTER, SWIFT and ALFERT 1951; WALKER and YATES 1952; DEELEY *et al.* 1954; and ROELS 1954) indicate, however, that reduplication occurs in late interphase just before the beginning of mitosis. In the present model, reduplication of the genonema during interphase is necessary to allow for prophase coiling without entanglement of the daughter strands as occurs in the giant chromosomes. If reduplication were to occur during prophase, after formation of the minor coil, it would be necessary to postulate a mechanism within the chromosome to cause paranemic coiling (SPARROW, HUSKINS and WILSON 1941). This would imply a genonema asymmetrical in cross-section and there is no direct evidence for such a configuration.

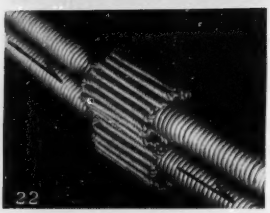
In the case of the salivary gland chromosomes the first duplication would occur while the chromosome strands were coiled in the prophase











Figs. 18—22

condition (Fig. 23a). Torsional forces within the original strand must be assumed to account for its coiled condition. Because the original strand would hold the daughter strand in position during replication, torsional forces would not necessarily be reproduced in the latter. Thus upon completion of duplication, the daughter strand would tend to loosen but would be held by the original strand due to the intertwining of the two (Fig. 23b). As a result, the strand bundle would loosen, both in length and diameter, with each replication. The longitudinal interval of the coils would increase with each division without appreciable torsional force being exerted upon the bundle of strands, but increase in overall diameter of the bundle would result in a tendency for the strand bundle to uncoil by rotation with consequent production of supercoils and supertwists in the thin chromosomes of the early stages (Fig. 24). With

Fig. 18 through 22 depict a proposed model for reduplication in the larval salivary gland chromosomes of *Drosophila*

Fig. 18. Model of a portion of one strand of a salivary gland chromosome or of an interphase chromosome before reduplication. In the center is shown one nucleoprotein granule with its associated strand material on the right (s). To the left (s^1) is the strand material of the neighboring granule (located out of the photograph to the left)

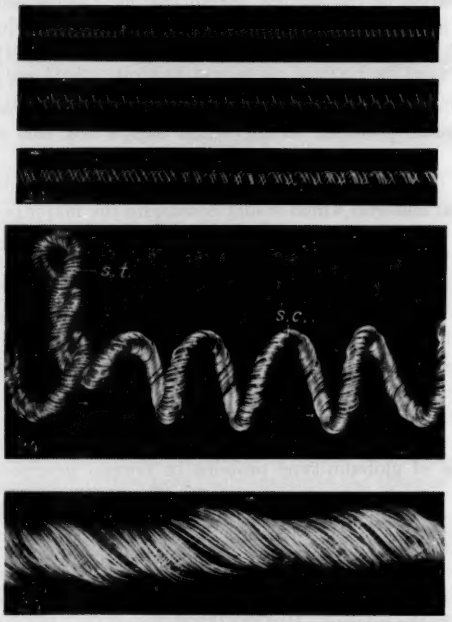
Fig. 19. The nucleoprotein granule has reduplicated itself with respect to molecular succession and configurational folding. Reduplication of all granules in the nucleus probably occurs synchronously

Fig. 20. The sheet-like daughter nucleoprotein is shown in the process of unfolding with each edge having passed through 90° of its 180° path

Fig. 21. The opposite edges of the sheet-like nucleoprotein have each rolled through 180° to form a new granule parallel and identical to the original granule

Fig. 22. The new nucleoprotein granule now synthesizes its strand material parallel to the chromosome axis to the neighboring new granule. Attachment of each strand to its neighboring granule is probably accomplished by the bridges of divalent cations

further replication and over-all increase in chromosome diameter the rigidity of the chromosome would increase and cause further uncoiling throughout its length with the subsequent elimination of the supercoils and supertwists (Fig. 25). Unequal torsion in the individual strands at



Figs. 23—25

Fig. 23 a—c. a. A model of part of a salivary gland chromonema in an early prophase coiled condition before the first endomitotic division. The nucleoprotein granules are not shown. b. A model of the parent and daughter chromonemata after the first division. The two strands are intertwined as a result of the coiled condition of the original strand. The strands, thus, are unable to separate. c. A model of a four strand chromosome following the second reduplication. The coils of the strands have loosened both in coil interval and diameter

Fig. 24. A model of an eight strand chromosome showing the production of supercoils (*sc*) and supertwists (*st*) resulting from the tendency of the strand bundle to uncoil by rotation throughout its length. The model is not truly representative of the actual condition since the chromosome would have a higher degree of polyteny at this stage

Fig. 25. A model of a sixty-four strand chromosome. In a living chromosome with this degree of polyteny, supercoils and supertwists would be present

this stage, however, would result in minor differences in path followed by individual strands throughout the length of the chromosome.

The configurations of the developing giant chromosomes of *Chironomus tentans* have been described in detail by BEERMANN (1952). That a

coiled condition of the strands is the basis of the developmental stages described by him is evident and, except perhaps for the "meander-stage" configuration, can be interpreted by the reduplication of coiled strands.

Reduplication of the strands within the giant chromosomes of any one nucleus appears to be synchronous with the final cells in *Drosophila* salivary glands being 1024-ploid (SWIFT and RASCH 1954).

There is indirect evidence that interphase chromosomes and salivary gland chromosome strands, in addition to reduplication, actively synthesize globular proteins. Observations supporting this view are as follows:

MULLER (1941) postulated the presence of an accessory or interchromonemal material which would constitute the major portion of the volume of the salivary gland chromosome. On the basis of the present work, the fibrils plus granules probably comprise from $^1/_{40}$ to $^1/_{20}$ of the volume. MAZIA (1950) found that crystalline pepsin caused a marked shrinkage of the chromosomes mostly in the interband areas. However, the removal of the interband material did not produce any breaks in chromosome continuity. Since pepsin affects proteins with neutral or acidic isoelectric points, the material removed from the chromosomes was probably globular proteins. The strand material would not be digested because of its fibrous state (MAZIA 1950). Evidence concerning the presence of globulin-type proteins in normal interphase nuclei is found in the work of KIRKHAM and THOMAS (1953) in which a major component of proteins extracted from nuclei with 14 M NaCl had characteristics of globulins.

Other evidence, however, involving the use of radioactive tracers, suggests that protein synthesis (globular or otherwise) does not occur extensively in the nucleus . HULTIN (1950) found that the rate of protein N replacement is not as rapid in the nucleus as that of other cell components, especially the microsomes. HAUROWITZ and CRAMPTON (1952) obtained similar results with I^{131} marked antigen.

In developing embryos, however, where metabolism is rapid and a relatively great amount of species specific protein synthesis would be expected, a greater uptake in the nucleus than in the cytoplasm of labeled glycine (SMELLIE, MCINDOE and DAVIDSON 1953; SIRLIN and WADDINGTON 1954), labeled orotic acid (FICQ 1954), P^{32}, N^{15}, S^{35}, and C^{14} (SMELLIE *et al.* 1953) has been found. Incorporation here is very slow in DNA, relatively slow in cytoplasmic RNA, but rapid in all nucleotides of nuclear RNA. This appears to support the postulate of HAUROWITZ and CRAMPTON (1952) and DOUNCE (1953) that the DNA exerts its specificity upon protein synthesis by acting as a template for RNA formation within the nucleus.

In view of the apparently conflicting observations and interpretations of the mechanism of nuclear control over protein synthesis and cell metabolism, and because of the lack of direct evidence concerning many aspects of chromosome fine structure, it is probable that several details of structure proposed in the present model are incorrect. However, this interpretation is not advanced as a finished description of chromosome fine structure but rather as an attempt to devise a model that will best fit the observations of numerous workers utilizing optical, biochemical, micrugical, genetic and electron-microscopical techniques.

Summary

The submicroscopic structure of the chromosomes of the larval salivary gland cells in *Drosophila melanogaster* has been studied by means of electron microscopy of thin sections.

The three fixatives used were buffered 1% osmium tetroxide, unbuffered Zenker's solution, and buffered formaldehyde in concentrations of 0·1% and 1·0%. It was found that formalin preserves the fine structure of the chromosomes better than the other fixatives.

Nucleoprotein is present in the chromosome in the form of granules 200 Å to 300 Å in diameter. The granules are connected together by strands of protein approximately 100 Å in diameter. The length of the strands between successive granules in the banded areas is 100 Å to 300 Å and in the interband regions it is 700 Å to 900 Å.

Heterochromatin differs from euchromatin in the absence or reduction in the amount of strand material.

The nucleolus is composed of an aggregate of granules 300 Å to 600 Å in diameter contained in an amorphous matrix. Within the nucleolus are a series of canals containing material similar in appearance to that of the euchromatic band material.

Submicroscopic granules are also present in the karyoplasm and in the cytoplasm surrounding the nuclear membrane. The cytoplasmic granules are more densely distributed near the nuclear membrane.

A model for chromosome structure is proposed.

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THE DEVELOPMENT IN THE HUMAN LIVER OF MULTIPLE DESOXYRIBOSE NUCLEIC ACID (DNA) CLASSES AND THEIR RELATIONSHIP TO THE AGE OF THE INDIVIDUAL*

By

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With 12 figures in the text

(Eingegangen am 3. Oktober 1955)

Introduction

The inability to account for the postnatal growth phases of the mammalian liver on the basis of a demonstrable increase in mitotic activity has led to the general belief that growth is due to the formation of the large uninucleate parenchymal cells present in the adult organ. Although JACOBJ (1925) thought that these cells resulted from an increase in chromosomal mass, but not number, the weight of opinion holds that they are polyploid (CLARA, 1930, 1931a; WILSON and LEDUC, 1948; HELWEG-LARSEN, 1952). It is now accepted that the adult liver parenchyma, of at least several mammals, consists of a mixture of diploid, tetraploid and octoploid cells. The evidence for polyploidy has come from the following sources:

a) Chromosome counts. Counts for mammalian liver are rare and the data that are present are often conflicting. For example, D'ANCONA (1941) using colchicine on regenerating rat liver, found the chromosomes dispersed and easily countable, and gave 23—24 as the diploid number and 91—97 as the octoploid number. But BIESELE (1944) reported counts of 40—42 (diploid) and 81—84 (tetraploid) from a squash preparation of rat liver. WILSON and LEDUC (1948) were unable to count chromosomes in rat liver but concluded, as did BEAMS and KING (1942) before them, that the comparative size of the mitotic figures is evidence enough for polyploidy. In 1931(b), CLARA described mitotic figures in rabbit liver and definitely believed them to be polyploid. BIESELE, POYNER and PAINTER (1942), counting plasmasomes as indicators of the number of times the chromatids had reduplicated in larger mouse liver nuclei, also report the presence of polyploid cells. Despite the discrepancies in the actual counts in rat liver, and the general sparsity of information on the subject, there seems to be at least enough evidence to conclude that the livers of some mammals contain nuclei with chromosome numbers which are multiples of the diploid.

b) Nuclear volumes. Perhaps the first indication of the existence of polyploid cells in normal mammalian tissues came from the study of nuclear volumes. In 1925 JACOBJ published his classical work in the course of which he found, for certain mammalian tissues, that the nuclear volumes form multimodal frequency distribution curves which show peaks bearing ratios to one another of approximately

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1:2:4:8. From this he postulated a rhythmical growth theory and suggested that the higher nuclear volume classes represented increased chromosomal masses. In 1952 HELWEG-LARSEN introduced new evidence of nuclear volume classes in mammals and statistically confirmed both his own and JACOBJ's data. In addition he presented a large number of references to nuclear volume classes in many different vertebrates and invertebrates. He stated that, at the time of writing, JACOBJ's interpretation of the Heidenhain growth formula was as yet unchallenged, that the doubling of a nuclear volume would invariably indicate the doubling of a nuclear mass.

However, it has been shown, in recent years, that the rhythmic growth of cells is not always accompanied by an increase in the amount of chromosomal material. This is particularly evident in the case of *Arvelius albopunctatus*, in which SCHRADER and LEUCHTENBERGER (1950) showed that the testis contains spermatocytes of three size classes whose nuclei bear the volume relationships 1:2:8, but which all contain the same amount of chromosomal material (DNA). This was the first evidence, at the cytological level, that accounted for the size differences in nuclear classes on the basis of a component other than the chromosomal mass. Data by other workers, at different levels and in different materials, have illustrated the same phenomenon (KOSTERLITZ, 1947; BODENSTEIN and KONDRITZER, 1948; ALFERT, 1950; PATTERSON and DACKERMAN, 1952).

While some nuclear volume classes have actually been shown to reflect rhythmic increases in chromosomal mass, it can be seen by the above that the employment of nuclear volumes as a reliable index to polyploidy must be approached with caution.

c) Microspectrophotometric determination of a stable chromosomal component. In light of the evidence gathered in recent years designating desoxyribose nucleic acid (DNA) as a primary and stable component of the chromosome (see SWIFT 1953 and LEUCHTENBERGER 1954 for recent reviews) perhaps the most convenient method for determining the degree of polyploidy in individual nuclei (in tissues which have already been established as polyploid, not polytene) is the measurement of the amount of DNA by microspectrophotometry. Ultraviolet microspectrophotometry, developed and introduced to biological research by CASPERSSON (1936) first presented such a tool for the quantitative determination of DNA in single nuclei. LEUCHTENBERGER et al. (1952) first employed this method on isolated rat liver nuclei to demonstrate the existence of three DNA classes having mean DNA contents closely related to one another by ratios of 1:2:4.

An adaption of the CASPERSSON method by POLLISTER and RIS (1947), utilizing the specificity of the Feulgen reaction for DNA (STOWELL, 1945), has been used extensively for the measurement of relative amounts of DNA in individual nuclei. With this apparatus MIRSKY and RIS (1949) first reported the presence of multiple DNA classes in the mammalian liver. They found adult rat liver to contain three nuclear DNA classes, the means of the classes giving approximate 1:2:4 ratios, with the lowest class corresponding to the diploid value for the animal. Other workers, using the same method or modifications of it, have reported similar results in rat and other adult mammalian livers (SWIFT, 1950; ALFERT, 1950; LEUCHTENBERGER *et al.*, 1951; MIRSKY and RIS, 1949, 1951). Of significance is the fact that no one has ever demonstrated the absence of these three DNA classes in any adult, normal, mammalian liver in which their occurrence has once been established. This fact, combined with the evidence for chromosome polyploidy in certain mammalian livers, both strengthens the validity of the microspectrophotometric method for the recognition of polyploidy in these tissues and testifies to the stability of the classes.

Statement of the Problem

SWIFT (1950) showed that polyploid cells arise in already differentiated tissues and increase in frequency with the age of the animal. LEUCHTENBERGER, LEUCHTENBERGER and DAVIS (1954), in a microspectrophotometric study of five human livers from children 3 months to 6 years of age and five livers of adults from 13 to 86 years of age, reported that in all the former only the basic diploid class (2 DNA) was present while all the latter presented the usual multiple DNA classes.

The concept of tissue growth by polyploidy is relatively new, for it was stated by DE BEER, as recently as 1924, that true growth is impossible when cells are unable to divide. While he recognized that an organ or tissue may increase in size also by an increase in size of the constituent cells, he emphasized that mitotic growth "is by far the more important and is often designated 'true' growth". Thus the present paper is an attempt to determine whether a correlation exists between the growth of a mammalian organ and the time of development of its polyploid cells, and whether or not this phenomenon is associated with demonstrable mitotic activity.

The human liver was selected for several reasons. First, the presence of multiple DNA classes in this organ has already been shown to be independent of a wide variety of normal and abnormal physiological conditions except age (LEUCHTENBERGER, LEUCHTENBERGER and DAVIS, 1954) and hereditary pituitary dwarfism (LEUCHTENBERGER, HELWEG-LARSEN and MURMANIS, 1954). Secondly, the growth stages of the human are well defined and well separated in time. Thirdly, because of the association of the Department of Cytochemistry with the Institute of Pathology, a large amount of human material was made available.

For the purpose of the study 1190 measurements were made in the livers of 37 individuals of both sexes, ranging in age from 2 weeks to 90 years.

Materials and Methods

The human liver specimens used in this study were from two sources: (1) paraffin imbedded tissues from the autopsy files of the Institute of Pathology, Western Reserve University and (2) fresh specimens from cases of accidental or unexplained deaths, from the Cuyahoga County Coroner's Office. Only those livers were finally selected for measurement which presented characteristically normal structure with no severe degenerative changes. The autopsy file specimens had been routinely prepared in the pathology laboratories: formalin fixed, washed in water, dehydrated in alcohols, cleared in xylol. The Coroner's office specimens were received in 10% formalin and subjected to the same treatment. Since it had been reported that DNA content per cell does not change for a considerable period after death (LEUCHTENBERGER, LEUCHTENBERGER and DAVIS, 1954) tissues were used which had been removed not longer than ten hours post mortem.

Relative amounts of DNA in individual nuclei were determined by microspectrophotometry of Feulgen-stained nuclei. The sections, cut at 12 μ to afford

sufficient uncut nuclei for measurement, were stained under carefully controlled conditions, as described by LEUCHTENBERGER (1950). Adjacent sections, unhydrolyzed, were used as controls. The microspectrophotometric apparatus is of the same type as that used by LEUCHTENBERGER, VENDRELY and VENDRELY (1951). The relative amount of DNA per nucleus was calculated by the following formula:

$$\frac{\text{Amount} = \text{Extinction} \times r^2 \text{ plug (in micra)}}{\text{\% Volume (plug to nucleus)}}$$

where the "plug" is the part of the nucleus selected for measurement. Only whole nuclei, and those as close to spherical as possible, were selected for measurement. Means are given for all similarly grouped nuclei and standard errors for all groups containing more than ten nuclei. Nuclear volumes were determined for all nuclei and are presented as the radius cubed in micra.

Since polyploid cells develop often in localized areas of the liver lobule, it is possible that by random measurement of 30 nuclei in each section, the polyploid nuclei may be missed. For this reason the sections were carefully studied with a low power objective before being taken to the microspectrograph. The areas to be measured were selected so that a representative number of nuclei in each size class would be included, and were noted accordingly.

The errors involved in microspectrophotometry have been discussed in detail several times (MOSES, 1952; SWIFT, 1953). Such errors, for example the one due to inhomogeneity of the absorbing substance (distributional error), are of such an order that they will not interfere with the validity of such measurements as have been made in this work, wherein the method has been employed for the detection of differences in DNA content on the order of 100% (SWIFT and KLEINFIELD, 1953). The close correlation of the DNA ratios given by the visible microspectrophotometric method with those obtained by biochemical and ultraviolet cytophotometric methods also attests to the validity of the former (LEUCHTENBERGER, 1954, 1954b).

Results

The DNA values and nuclear volume determinations in individual human liver nuclei are presented in the histograms of Figs. 1–9, with the subjects arranged according to age. The mean value for all the 2DNA nuclei measured is 2.61 arbitrary units and is in good agreement with the value of 2.81 given by LEUCHTENBERGER, LEUCHTENBERGER and DAVIS (1954) for diverse human tissues. The individual means of all the measured nuclei composing the three classes are 2.61 (2DNA), 5.07 (4DNA) and 9.92 (8DNA), closely approximating the theoretical 1:2:4 ratio. The deviations from this ratio in individual livers may be attributed in part to the inherent errors of the method but there is also good reason to believe that an actual inconstancy in the amount of chromosomal material from nucleus to nucleus exists in mammalian somatic tissues (HUSKINS, 1947; HUSKINS *et al.*, 1948a, 1948b, 1950; MOORE, 1950; TIMONEN and THERMAN, 1950; BEATTY, 1954). In addition to contributing to the deviations from the theoretical means, this inconstancy may

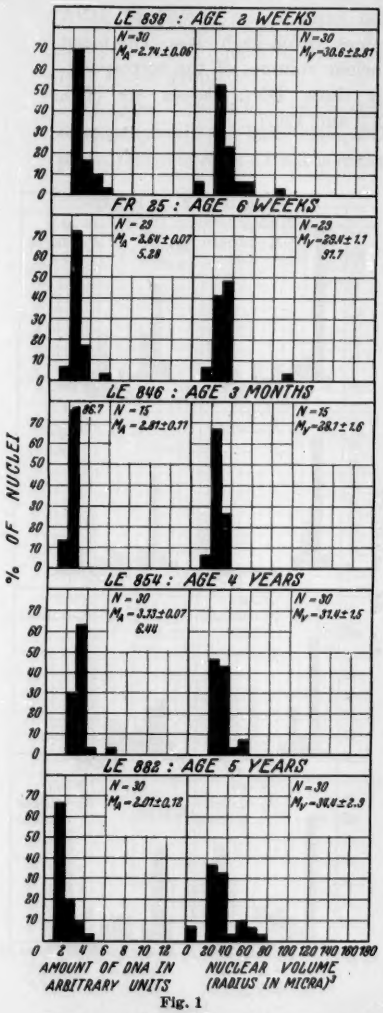

Fig. 1

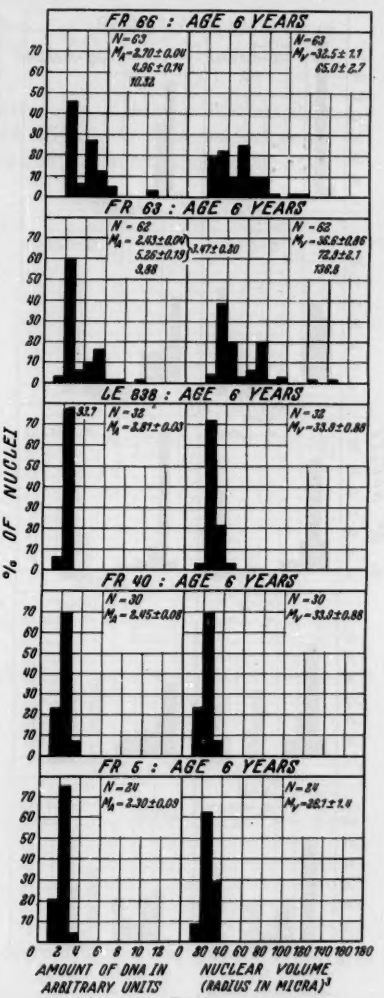

Fig. 2

Fig. 1. Age group 1, 2 weeks to 5 years, inclusive. Left, amount of DNA in individual liver parenchyma nuclei (by microspectrophotometry) Right nuclear volumes (radius in micra)³

Fig. 2. Age group 2, 6 to 10 years, inclusive (continued in Figs. 3 and 4). Left, amount of DNA in individual liver parenchyma nuclei (by microspectrophotometry). Right, nuclear volumes (radius in micra)³. (Figs. 1 to 9: M_A = mean amount of DNA; M_V = mean nuclear volume)

also explain the spread of DNA values within each class. The mean nuclear volumes of the corresponding three DNA classes are 35.5, 72.9 and 129.7.

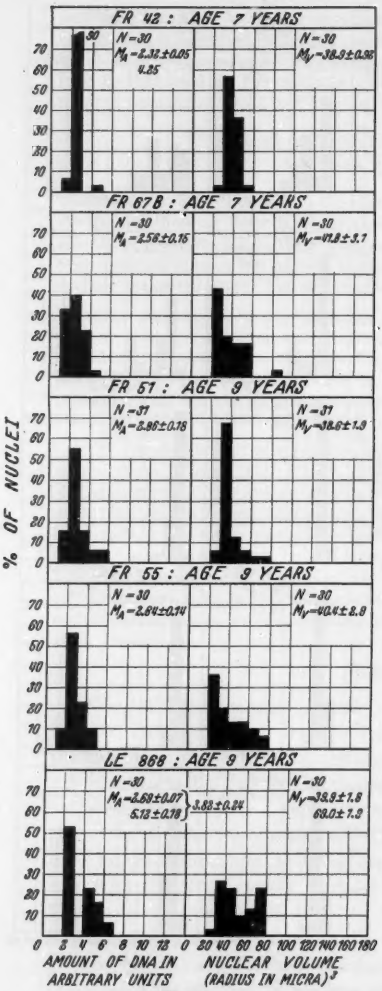
FR 42 : AGE 7 YEARS
FR 678 : AGE 7 YEARS
FR 51 : AGE 9 YEARS
FR 55 : AGE 9 YEARS
LE 868 : AGE 9 YEARS
% OF NUCLEI
AMOUNT OF DNA IN ARBITRARY UNITS
NUCLEAR VOLUME (RADIUS IN MICRA)³

Fig. 3

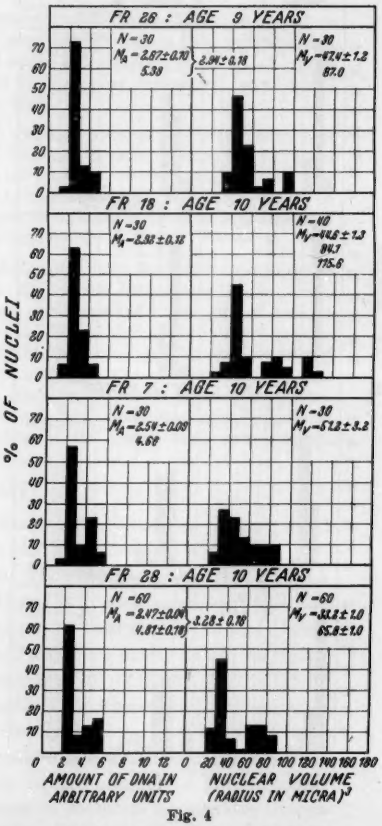
FR 26 : AGE 9 YEARS
FR 18 : AGE 10 YEARS
FR 7 : AGE 10 YEARS
FR 28 : AGE 10 YEARS
% OF NUCLEI
AMOUNT OF DNA IN ARBITRARY UNITS
NUCLEAR VOLUME (RADIUS IN MICRA)³

Fig. 4

Fig. 3. Age group 2, 6 to 10 years, inclusive (continued). Left, amount of DNA in individual liver parenchyma nuclei (by microspectrophotometry). Right, nuclear volumes (radius in micra)³

Fig. 4. Age group 2, 6 to 10 years, inclusive (continued). Left, amount of DNA in individual liver parenchyma nuclei (by microspectrophotometry). Right, nuclear volumes (radius in micra)³

On the basis of the relative presence or absence of DNA classes in the liver, the subjects in this study have been arbitrarily divided into four major groups.

1. Ages 2 weeks to 5 years, inclusive. All of these individuals show essentially only the basic class, 2DNA (Fig. 1). An occasional single representative of class 4DNA is encountered, but microscopic examination

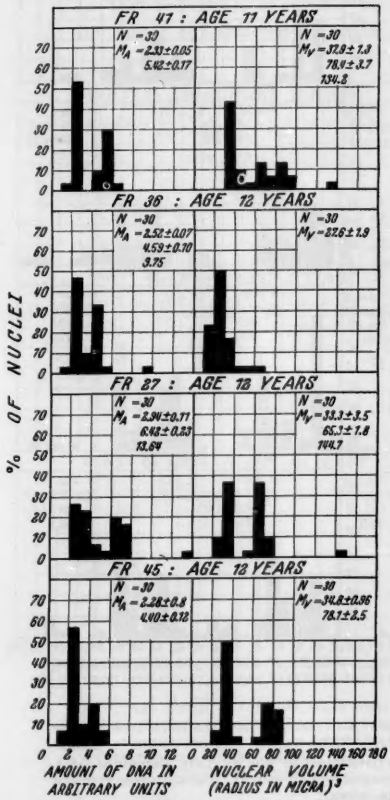
FR 47 : AGE 11 YEARS
FR 36 : AGE 12 YEARS
FR 27 : AGE 13 YEARS
FR 45 : AGE 13 YEARS
% OF NUCLEI
AMOUNT OF DNA IN ARBITRARY UNITS
NUCLEAR VOLUME (RADIUS IN MICRA)³

Fig. 5

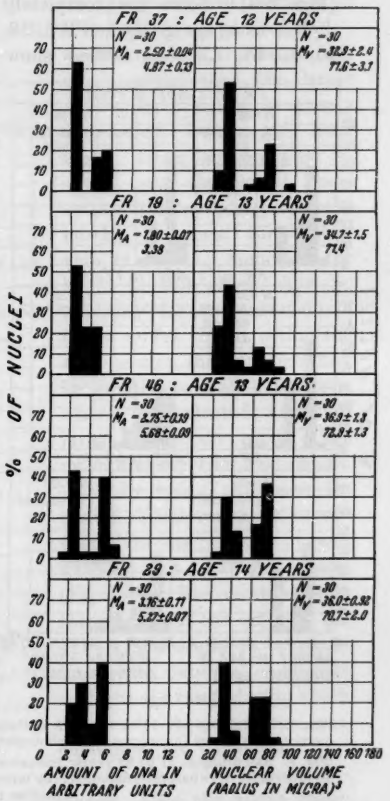
FR 37 : AGE 12 YEARS
FR 19 : AGE 13 YEARS
FR 46 : AGE 13 YEARS
FR 29 : AGE 14 YEARS
% OF NUCLEI
AMOUNT OF DNA IN ARBITRARY UNITS
NUCLEAR VOLUME (RADIUS IN MICRA)³

Fig. 6

Fig. 5. Age group 3A, 11 to 14 years, inclusive (continued in Fig. 6). Left, amount of DNA in individual liver parenchyma nuclei (by microspectrophotometry). Right, nuclear volumes (radius in micra)³

Fig. 6. Age group 3A, 11 to 14 years, inclusive (continued). Left, amount of DNA in individual liver parenchyma nuclei (by microspectrophotometry). Right, nuclear volumes (radius in micra)³

at low power clearly shows that this is atypical for the tissue as a whole. It has, in the past, been observed that many characteristically diploid tissues will occasionally present a tetraploid cell.

2. Ages 6 to 10 years, inclusive. As a group, the livers of these children do not show the uniformity in DNA classes seen in the other groups (Figs. 2–4). Some are completely diploid as in age group 1 (FR 5, 18, 40, 42, 51, LE 838). Others show

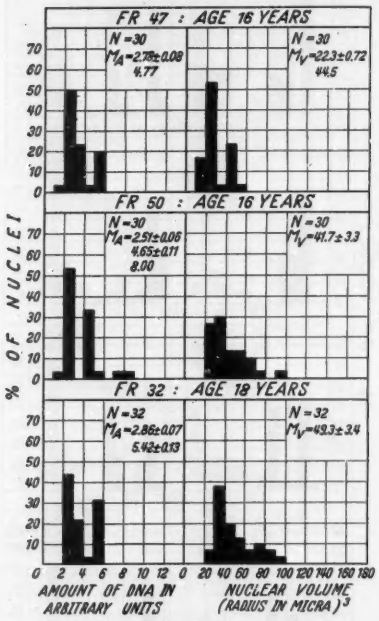

Fig. 7

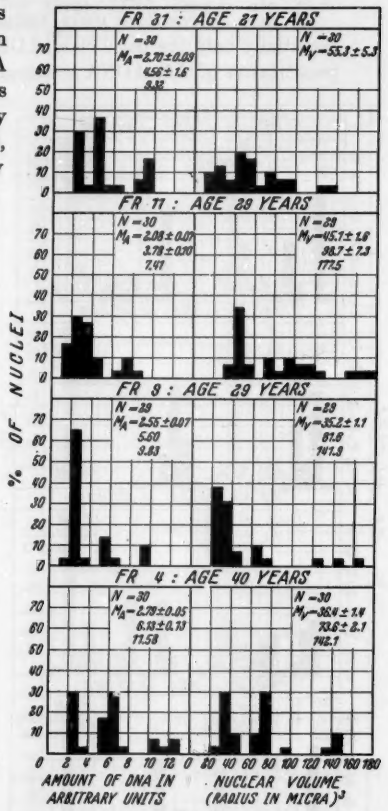

Fig. 8

Fig. 7. Age group 3 B, 16 to 18 years, inclusive. Left, amount of DNA in individual liver parenchyma nuclei (by microspectrophotometry). Right, nuclear volumes (radius in micra)³

Fig. 8. Age group 4, 21 to 90 years, inclusive (continued in Fig. 9). Left, amount of DNA in individual liver parenchyma nuclei (by microspectrophotometry). Right, nuclear volumes (radius in micra)³

some DNA values which approach and even exceed the 4 DNA class but which do not show distinct peaks at this value (FR 26, 28, 53, 63, 67 b, LE 868). Since FR 26, 28, 63 and LE 868 show at least a tendency

toward the formation of a peak near the 4 DNA value, means are given for two classes as well as for all the nuclei grouped as one.

FR 66 shows distinct peaks near both the 2DNA and 4DNA values but frequent mitotic figures were found in this liver. Mitoses in this group are considered in the discussion.

FR 7 is the only subject in this group that shows the presence of distinct 2 DNA and 4DNA classes in the absence of mitoses. In this respect it is similar to the next age group and, because of the small age difference, could well be considered a part of it.

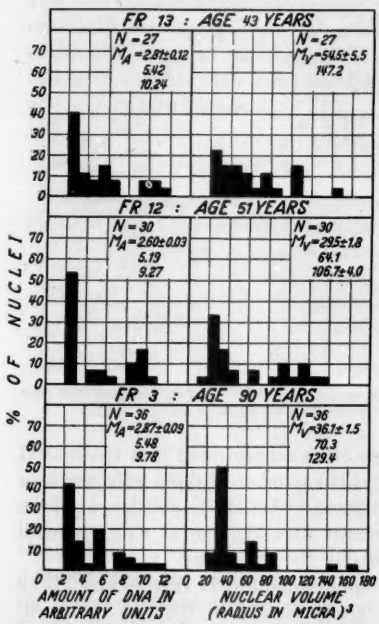

Fig. 9. Age group 4, 21 to 90 years, inclusive (continued). Left, amount of DNA in individual liver parenchyma nuclei (by microspectrophotometry). Right, nuclear volumes (radius in micra)³

Microscopic studies in this group reveal that the larger nuclei are not always evenly distributed within the liver lobule, being concentrated in some near the portal areas and/or the periphery of the lobule, and near the central vein in others. Some of the livers in this group show no tendency for concentration of larger nuclei. Even within the same section the situation may be greatly variable. However, where concentrated areas of larger nuclei do occur, they most often lie near blood vessels.

3. A) Ages 11 to 14 years, inclusive. These, without exception, show the presence of both 2DNA and 4DNA classes (Figs. 5–6). The most striking feature is that the onset of the 4DNA class seems to be correlated with puberty, or at least with the age range during which most of the secondary sex characters are developed.

B) Ages 15 to 18 years, inclusive. Like the above group *(3a)* all these individuals show the presence of the 2DNA and 4DNA classes (Fig. 7). The subdivision of group 3 was made only to emphasize that the first definite establishment of the tetraploid nuclei occurs during the beginning of this period. Diploid and tetraploid nuclei are apparently

maintained until about the twentieth year without the introduction of a well defined 8 DNA class. While an occasional 8 DNA nucleus is encountered throughout the 11–18 year range, microscopic examination again shows this to be atypical for the tissue as a whole.

With increasing age, the larger nuclei are found not to be concentrated in localized areas of the lobule, but distributed evenly within it.

4. Ages 21 to 90 years, inclusive. All of the individuals in this group present, without exception, three nuclear classes: 2DNA, 4DNA and

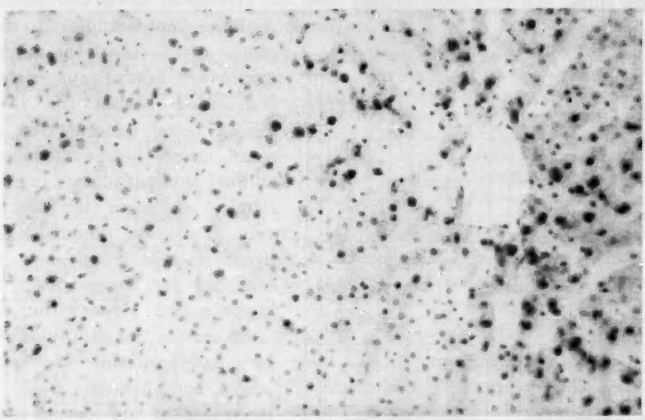

Fig. 10. Section of adult human liver showing nuclei of 8 DNA content concentrated near the central vein. Magnification ×600

8DNA (Figs. 8–9). 8DNA cells are often concentrated near the central vein (Fig. 10) and it is evident that the number of these cells increases with increasing age. Of interest is the fact that the establishment of the 8DNA class appears to be correlated with the age at which general body growth ceases, at approximately the 20th year.

From their histograms it may appear that FR 11 (Fig. 8) and FR 19 (Fig. 6) consist only of a single group instead of the two indicated. This is due to the fact that, in FR 11 for example, the 2DNA class consists of high 1 and low 2 values, while the 4DNA class consists of high 2 and low 3 values. The 2 values of both groups thus fall together, obscuring the separation of the classes. FR 19 presents a similar case.

With reference to the nuclear volume classes, it can be seen that the means, in most cases, fall into groups which closely parallel the DNA classes. There are, however, some striking exceptions. For example, FR 18 (Fig. 4) shows only a single DNA class although three nuclear volume classes are observed. FR 36 (Fig. 5) shows two distinct DNA

classes and only a single nuclear volume class. Other discrepancies can be noted. The number of nuclei composing the individual nuclear volume and DNA classes is rarely identical, even though the same nuclei were used for each determination. Furthermore, there appears to be a considerably wider range of values within each volume class than within each DNA class. As a result the DNA classes are more distinctly defined.

Most of the sections used for DNA measurements were studied for mitotic figures. Several of the livers in the age group 6–10 (FR 18, 63, 66) presented frequent mitotic figures (as has been reported by LEUCHTENBERGER, LEUCHTENBERGER and DAVIS, 1954). However, no correlation was noted between the presence of mitoses and the presence of nuclei with increased amounts of DNA. All the remaining livers in which the measurements were made showed either no mitoses in the parenchyma, or very rare instances.

Discussion

a) Polyploidy as a phenomenon of tissue growth

If one refers to the curve depicting increase in human liver weight with age (Fig. 11, computed from data by BOYD, 1941) it can be seen that

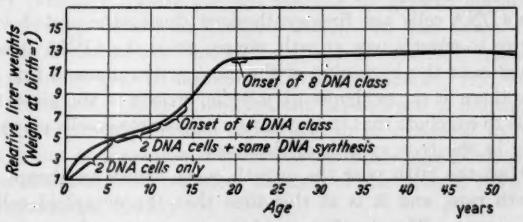

Fig. 11. Increase in human liver weight with age (after BOYD, 1941). The onset of the multiple DNA classes is also indicated

although the liver gains weight for several years after birth, there is a falling off of the *rate* of weight increase. From the times of onset of the polyploid classes (superimposed on the growth curve) it is seen that, during the first five years, the parenchyma consists only of diploid (2DNA) cells. It still remains to be answered how the liver continues to gain weight, and even to increase its cell number (CLARA, 1931a), in spite of the absence of visible mitoses and the formation of polyploid cells.

The next distinguishable phase of the growth curve occurs approximately during the years 6 to 10, at which time it appears that the rate of weight increase begins to rise. It is also during this period that some livers show signs of DNA synthesis, and while there is also some mitotic

activity, the two phenomena appear not to be related. SWIFT (1950b) and others (see SWIFT, 1953 and LEUCHTENBERGER, 1954 for reviews) showed for mitotic tissues that DNA is synthesized to double its basic amount prior to mitosis. This synthesis occurs most frequently during interphase and is reflected in the microspectrophotometric data by the presence of DNA values scattered between the basic amount for the tissues and double that amount. It seems not unreasonable to assume that DNA would also be gradually synthesized in a tissue preparing to grow by endomitosis, as is the case, here, since polyploid cells also acquire multiple amounts of DNA. Thus the DNA values in this group may be interpreted as reflecting such a period of DNA synthesis in preparation for the establishment of the class 4DNA.

By the 11th or 12th years, the liver enters into an almost linear phase of weight increase which continues until about the 20th year. The 4DNA class is established at the beginning of this period, apparently in the absence of mitosis. It is certainly true that the formation of a number of polyploid cells in a tissue will contribute to a weight increase of that tissue, although in this case it must still be determined whether the observed weight increase can be accounted for by the percentage of polyploid cells formed.

Since 4DNA cells are first synthesized during the period when the liver begins to enter a new growth phase, since the 4DNA class is well established near the beginning of the most active phase of this growth, and since there is no evidence of mitotic growth at this time, it seems justifiable to conclude that the formation of tetraploid cells plays at least some role in the true growth of the liver.

At about the 20th year the growth curve shows an abrupt drop in the growth rate, and it is at this time that the octaploid cells make their appearance. The significance of the appearance of a new polyploid class at a time usually associated with the cessation of general growth presents a difficult problem. The possibility that the octaploid cells represent an aging or degenerative process should be considered, but there seems to be no evidence to support such an hypothesis. If they were part of a degenerative phenomenon, one would expect that the number of octaploid cells would be significantly increased with age. To the contrary, their number seems to remain fairly constant from the fiftieth year on (unpublished preliminary observation). Secondly, from what is known of polyploidy in plants and other animals, there are many instances and suggestions that polyploid cells are associated with a particular function and, while it is much too early to generalize, all the evidence thus far favors the latter point. Thus in insects the polyploidy of glandular and other secretory cells is quite general (see GEITLER, 1953 for recent review), for example in the aquatic insect *Corixa*

punctata, polyploid cells seem to be associated with bristle formation (LIPP, 1953). Among plants, the succulence of the leaf mesophyll in *Kalanchoe blossfeldiana* is enhanced the higher the degree of polyploidy (VON WITSCH and FLÜGEL, 1952). Polyploid forms are also more resistant to unfavorable environments, being more widespread and resistant to arctic and dry climates (VANDEL, 1946).

The possibility must also be considered that octoploid cells are formed as a compensatory mechanism for the death of other cells. It would not be surprising to find such a mechanism in a tissue which appears to have lost its capacity for *normal* mitotic growth at an early age.

In summary, it has been shown that at least part of the postnatal growth of the human liver occurs by the formation of tetraploid cells at puberty. The role of the octoploid cells is obscure at this time but several possibilities have been offered to explain their presence.

b) Polyploidy and the anterior pituitary growth hormone

In addition to the role of polyploidy in the growth of the liver recent work has shed some light on the mechanism responsible for its expression. Nuclear class series, invariably present in normal mice, are absent in hereditary pituitary dwarfs but are restored by growth hormone substitution therapy (HELWEG-LARSEN, 1952). In the same material multiple DNA classes also fail to form but are similarly restored by anterior pituitary growth hormone (LEUCHTENBERGER, HELWEG-LARSEN and MURMANIS, 1954). The apparent dependence of multiple DNA classes on the growth hormone seems also to be extended to humans. A recent group of measurements on tissues of a single adult pituitary dwarf has demonstrated complete absence of the normal DNA classes (LEUCHTENBERGER, 1954c).

SELYE (1947) reports the average age of puberty to be about 14 for the female and 17 for the male in the United States and Canada. Puberty, in this sense, however, is the age at which reproduction becomes possible. Clearly the variation from individual to individual is great and many secondary sex characters indicative of puberal changes are completed before reproduction is possible. Puberty, in its more common context, is often associated with the ages of 11–14. The first puberal changes, however, can be seen to begin as early as 7 years of age, at which time the body contours of boys and girls commence to deviate from a common pattern, presumably under hormonal influence. Thus the synthetic activity of DNA in the 6–10 year group not only follows closely the change in the growth curve characteristic of these ages but also correlates well with the hormonal changes associated with puberty. Although it has never been demonstrated for the human that there is a special activity of the

anterior pituitary growth hormone at puberty, the changes in the general growth pattern suggest such an activity. If this is the case, then the appearance of polyploid cells in the liver at this time adds further evidence of the dependence of polyploidy on the growth hormone. In any case, the development of polyploid cells in the liver at puberty, combined with the necessity of the growth hormone for the expression of polyploidy, suggests that there may be some special activity of the growth hormone at this time.

One of the more interesting findings by LEUCHTENBERGER, HELWEG-LARSEN and MURMANIS (1954) is that, although the growth hormone restores DNA classes to the dwarf mouse, the body weight remains well below that of the normal litter mates. They discuss the possibility that the hormone may be concerned only with the formation of *multiple* DNA classes and not with the basic class, without excluding the latter possibility. The fact that the human liver grows rather continuously from birth, and then enters into a puberal growth phase, characterized by polyploidization, and apparently dependent on the growth hormone, may support this hypothesis. In other words, with respect to certain tissues, *general* growth may be independent of the growth hormone, while certain specialized phases of growth may be dependent on it. One of the more generalized effects of the growth hormone in vertebrates is in relation to the longitudinal growth of bones. Yet in amphibia, reptiles and young rats, growth in length is independent of this hormone. Clearly, growth is a complex phenomenon, dependent on different factors at different times. Since the growth hormone does not exert the same influence on the same tissues in different vertebrates, nor on different tissues in the same animal, it would not be surprising to find that it can stimulate growth by endomitosis in certain mammals and not body growth in general.

The position of polyploid cells within the liver lobule has demanded some attention in the past. JACOBJ (1925) reported that cells with large nuclei lie mainly in the intermediate zone of the lobule. On the other hand, MÜNZER (1923) and SULKIN (1943) found the polyploid cells more concentrated near the central vein. In the present study it was reported that the larger nuclei were usually more concentrated in small areas of the lobule, most often near the portal area and at the periphery, and less frequently near the central vein. However, whenever such concentrations of polyploid nuclei occur it is always near an area of high vascularity. Thus far the only exception to the spatial association of polyploid nuclei and blood vessels is reported by JACOBJ. Since it was found in the present study that the situation is greatly variable from lobule to lobule within the same section, and also from liver to liver, this apparent discrepancy may be explained by the fact that JACOBJ used only one mouse

and one rat. Perhaps a reconstruction of an entire liver lobule would clarify the inconsistencies found in the study of single sections. The majority of observations, indicating that the most active seat of growth is near an area of high vascularity, may support the concept of a blood-circulating stimulus for polyploidy, but it is clear that these findings must await further confirmation.

c) Mechanism of polyploidization

Various mechanisms have been proposed for the formation of polyploid cells in the mammalian liver. WILSON and LEDUC (1948) report that the liver may begin to grow by a mitotic process which later yields to one exclusively endomitotic. On the basis of cytological observations in mouse liver stimulated to mitotic activity by various experimental procedures, they postulate a progressive suppression of the mitotic process. The first step would be the delay and failure of cytoplasmic division, resulting in a binucleate cell. The next step is represented by the occurrence of the telophases without spindle formation, the prometaphase proceeding to telophase without division. The endomitotic process is initiated when the chromosomes, in the contracted condition seen at mid-mitosis, are reduplicated without nuclear membrane breakdown. The final stage would be the division of the chromonemata, without condensation, in the "resting" nucleus, a process which WILSON and LEDUC have designated "cryptomitosis". This term is unfortunate for, while it was earlier thought that no diagnostic structural changes occurred in this type of endomitosis, adequate cytological study has shown that fine structural changes do take place (see GEITLER, 1953 for review).

It is doubtful that the "mitotic suppression" described by WILSON and LEDUC occurs in the normal human liver. The possibility cannot be denied that the mitotic figures encountered in some of the 6–10 year group may represent the beginning of such a process. The difficulty with this hypothesis lies in the occurrence within this age group of cases in which DNA synthesis apparently goes on without visible mitotic activity. The absence of mitoses in these livers, however, does not exclude the possibility that such activity may be periodic, having taken place before the liver was fixed. Even with this objection satisfied, the fact still remains that none of the intermediate stages described have been observed. It is thus suggested that the chromosomal material in the normal human liver is reduplicated without obvious condensation and within the nuclear membrane.

BEAMS and KING (1942), on the basis of observations on regenerating livers, have proposed that polyploid nuclei arise only by mitotic processes

in which spindles of binucleate cells fuse. The general absence of mitotic figures in the normal human liver during the development of 4 DNA nuclei is apparently incompatible with this theory.

Still other possible mechanisms for the origin of polyploid cells in mammalian liver have been suggested. Thus PFUHL (1938), and later DAWSON (1940) postulate that large single nuclei in the liver may be formed by the fusion of the two nuclei of constantly present binucleate cells. The consistent appearance of dumbbell-shaped nuclei in the liver (Fig. 12) has been cited as evidence of this process (DAWSON, 1940) but these same nuclei have also been interpreted as indicative of amitotic division, perhaps leading to binucleate cells (MÜNZER, 1925). In neither case would these phenomena, in themselves, explain liver growth.

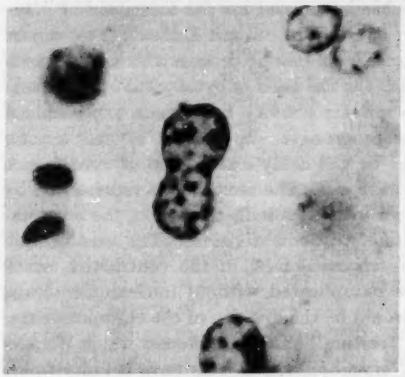

Fig. 12. A bilobed nuclear figure commonly found in normal human livers. Magnification × 1800

The tentative rejection of the above proposed mechanisms for the formation of polyploid cells in the liver are based, of course, on the inability to demonstrate mitotic phenomena in the normal organ, and also on the inadequate evidence for either fusion or amitosis of liver nuclei. Should any of these phenomena be shown to exist in the normal liver, it is obvious that a whole host of new possibilities will be opened, both to explain the formation of polyploid cells and the mechanism of growth from puberty on. Until this happens, it seems that simple endomitosis remains the most feasible explanation for the formation of polyploid nuclei in the human liver.

d) Significance of polyploid cells in mammalian tissues

Little is known of the significance of polyploid cells in mammalian tissues and this work, unfortunately, can shed little light on the subject. If the development of polyploidy is dependent, at least in part, on the anterior pituitary growth hormone, and if polyploidy is the method by which the liver grows, the question of major significance still remains unanswered: are polyploid cells only quantitatively different from their diploid neighbors, or is there a basis for qualitative differentiation?

Until only a few years ago there was no basis on which qualitative differences between nuclei of the same organism could even be discussed. In 1947, though, HUSKINS was able to point out that "The dogma of chromosome and gene identity in all cells was one barrier to the acceptance of the possibility. It is swept aside by the total data now available on polysomaty." He then goes on to show that the concept of the gene as a unitary organic molecule was another barrier to the acceptance of qualitative differences between nuclei and relates how that block has been reduced by the evidence that the "geneticists' supposedly unitary chromatid is actually two or more stranded". With the door open to such considerations of nuclear differentiation, others have been able to suggest means through which such differentiation could take place. GEITLER (1948) considers endomitosis to be a simplified process compared with mitosis and points out that it inhibits the function of a tissue to a lesser degree. Growth by polyploidy, he reports, will produce a nuclear-cytoplasmic relationship different from that of the diploid cell and will thus create different physiological conditions.

SCHULTZ (1952) also considers the problem in some detail. He points out that it is difficult to conceive that "the activities of the different, genically controlled, cellular enzymes remain in constant proportion to each other as the amount of each enzyme synthesized increases". The rationale for this statement lies in the fact that "the curve relating activity to amount of enzyme will have a different slope for different enzymes; and the flat portion of the curve, in which an increase in amount of enzyme no longer results in an increase in that activity in the cell, may be attained with different amounts for the various enzymes. Thus, even though the actual amounts of the enzymes synthesized may increase in proportion with the endomitotic index for all enzymes, divergence in the enzymatic activities in the different cell types may occur as a result of the endomitotic process automatically". He goes on to point out that at least one case is known in which there is a differential multiplication of chromosome regions in polyploidy and thus another basis for qualitative differentiation of nuclei.

The inactivation, or detoxification, in the human liver of testosterone and estrogen, apparently present in greatly increased amounts at puberty, furnishes the only clue to the significance of the development of polyploidy at this time. As cytochemical techniques improve and new ones are developed, perhaps the problem will be profitably directed toward the detection of qualitative differences, if such exist, among nuclei exhibiting different degrees of polyploidy in the same tissue. Through such differences it is hoped that some insight may be gained into the reasons for growth by polyploidy in some tissues and growth by mitosis in others.

Summary

Amounts of desoxyribose nucleic acid (DNA) in individual liver nuclei were determined microspectrophotometrically for 37 humans ranging in age from 2 weeks to 90 years. It was found that:

1. Until the age of about 6 the liver contains only diploid (2DNA) nuclei.

2. During the years 6–10 there is evidence of DNA synthesis in some nuclei.

3. During the years 11–14 a definite tetraploid (4DNA) class is established and until the age of about 20 the liver contains diploid and tetraploid nuclei.

4. At about the age of 20 an octaploid (8DNA) class is established and all three DNA classes coexist in the liver until death.

It is concluded that:

1. The growth phase of the human liver at puberty can be explained, at least in part, as growth by the formation of polyploid cells as opposed to growth by mitosis.

2. The data presented are consistent with the hypothesis that the development of polyploidy in mammalian organs is dependent on the anterior pituitary growth hormone.

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INTERFEROMETRIC DRY MASS AND MICROSPECTROPHOTOMETRIC ARGININE DETERMINATIONS ON BULL SPERM NUCLEI WITH NORMAL AND ABNORMAL DNA CONTENT*

By

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With 6 figures in the text

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The use of the interference microscope for dry mass determinations of cell structures (MELLORS 1953, 1955; BARER 1952, 1955; DAVIES et al. 1954) has opened new pathways for the study of the chemistry of cellular components. To the cytochemist who attempts a quantitative evaluation of chemical constituents in *single* cells or cell parts by microspectrophotometry (for review, see POLLISTER 1950, DAVIDSON 1953, SWIFT 1953, LEUCHTENBERGER 1954, 1954a), a standard of reference such as the dry weight is of special interest because he can now relate the quantity of intracellular substances to the total dry weight of the cell structure under investigation. The reference of analytical data to dry mass is of course a customary method which the biochemist has been using for a long time but there is a striking difference between the two approaches which has to be considered, especially when results from both methods are compared with each other and interpreted.

The interference microscope permits the dry weight determination of a *single* cell structure, such as the nucleus or nucleolus *directly* in situ under the microscope so that variations from cell to cell can be easily assessed. In contrast, even under the most favorable conditions, the biochemist, using the most sensitive balances, can determine the dry weight only on a cell population which consists of at least several million cells. Consequently the value for a single cell has to be *computed* from the result obtained on such a cell population and must of necessity be only an *average value* which does not give any information on the variability from cell to cell. The evaluation of such an average value is especially difficult and may lead to an erroneous interpretation, if one deals with an inhomogeneous cell population derived from tissues in which polyploidy, mitosis or pathological changes are present (LEUCHTENBERGER 1954, 1954b).

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One of the problems in which we have been engaged for a long time and where interferometric dry mass determinations seemed of interest concerns the difference in desoxyribose nucleic acid (DNA) content between the spermatozoa of fertile and infertile males. In previous extensive microspectrophotometric DNA studies which we have done on human spermatozoa, it was found that the amount of DNA in the spermatozoa of fertile males is very constant and corresponds to the haploid DNA value, while the DNA content in the spermatozoa from infertile males is variable and significantly lower than that of the fertile males, although the cytological appearance of the spermatozoa with the normal and abnormal amounts of DNA was identical (Leuchtenberger et al. 1953, 1954c, 1955, 1955a). Essentially the same results were obtained in cattle; that is, fertile bulls have a constant haploid amount of DNA, while sterile bulls have a deficient DNA content in their spermatozoa[1].

In the present report, we are not concerned with the problems of fertility or infertility, but only with the differences in DNA content in the spermatozoa and the relationship of these differences to other chemical components in the sperm nuclei. Since the main chemical constituents of bull sperm nuclei are DNA linked to a basic protein with a conspicuously high amount of arginine (Vendrely and Vendrely 1953, Mann 1954), an investigation of the arginine content and of the dry mass of sperms with normal and deficient DNA contents seemed justified. It was hoped that such studies might help in elucidating some of the pertinent questions which arose in connection with this DNA deficiency.

1. Is the DNA the only constituent which is reduced in its content or is the basic protein, as represented by arginine, also involved?

2. Do the dry masses of sperm nuclei containing low amounts of DNA differ from those with the normal haploid amount?

In the following, the results of DNA, arginine and dry mass studies on 4000 individual bull spermatozoa are presented. The work was done on bull spermatozoa because their cytologically homogeneous appearance is especially favorable for microspectrophotometric DNA, arginine and interferometric dry mass determinations. Furthermore, a comparison with the biochemical data which are available on the DNA (Leuchtenberger et al. 1951) and arginine (Vendrely and Vendrely 1953) content in bull spermatozoa confirms the validity of the microspectrophotometric determinations.

[1] A detailed report on the DNA findings correlated with a fertility analysis and breeding results in cattle will be published elsewhere in cooperation with Dr. Keith Huston of Kansas State College. It is to be emphasized that that report is concerned with cattle of normal size. Dwarfism in cattle is the subject of another paper (by Leuchtenberger, Schrader, Hughes-Schrader, and Gregory) now in preparation.

Material and methods

In order to carry out these studies, seminal fluids from 85 fertile bulls were secured from the Virginia, Northern Ohio, Central Ohio and Kansas Breeding Stations. For the DNA and arginine determinations, smears of the seminal fluids were made, fixed immediately in Carnoy and stained by the Feulgen-Fast Green technique as previously described (LEUCHTENBERGER 1950, SCHRADER and LEUCHTENBERGER 1950), but with the modification that the Feulgen and Fast Green stains were both carried out consecutively on the same slides.

The specificity of the Feulgen reaction for DNA can be considered as established (LEUCHTENBERGER 1954, 1954a). However, the use of Fast Green for the demonstration of arginine in bull spermatozoa warrants an explanation. It was shown by CHAPMAN et al. in 1927 and FRANKEL-CONRAT et al. in 1944 that a stoichiometrical relation exists between the amount of free basic groups in various proteins and their capacity to bind acidic dyes. This method has been successfully applied to tissue sections by SCHRADER and LEUCHTENBERGER (1950) and DEITCH (1955). Since bull sperm nuclei consist predominantly of desoxynucleoproteins and have an extremely high content of arginine [25.47 mgm. per 100 gr. dried ash- and lipid-free spermatozoa (MANN 1954)], it seems justified to use the Fast Green stain as an indication of the quantity of arginine in bull sperm nuclei. While some of the Fast Green staining might be due to the presence of small amounts of histidine and lysine, there are no other free amino groups in the sperms which could combine with the Fast Green stain. This is due to the fact that the Fast Green stain was done on the same slide following the Feulgen stain, that is, after the purine and pyrimidine bases have been split off by the acid hydrolysis.

The amounts of DNA and arginine were determined in individual spermatozoa microspectrophotometrically as previously described (LEUCHTENBERGER et al. 1953, 1954). Both the DNA and arginine measurements were made on the same sperm. The validity of microspectrophotometric methods has been repeatedly demonstrated (for review see POLLISTER 1950, DAVIDSON 1953, SWIFT 1953, LEUCHTENBERGER 1954a). The amounts for DNA and arginine are given in arbitrary units and also in absolute values. The former are given for the convenience of other workers in the field who use the same arbitrary units. The absolute amounts for DNA are based on previous studies (LEUCHTENBERGER et al. 1951) LEUCHTENBERGER et al. 1952, LEUCHTENBERGER et al. 1952) in which the biochemical analysis when compared with ultraviolet and Feulgen microspectrophotometry established that a factor of 2×10^{-9} mgm. will convert arbitrary units to absolute amounts. For the conversion of the arbitrary units of arginine, a comparison with the biochemical value obtained by VENDRELY and VENDRELY (1953) showed that 1×10^{-9} mgm. will convert the arbitrary units to absolute amounts. In each case, a statistical analysis was carried out and each mean value which was obtained on a number of sperm nuclei larger than 10 is given with its standard error.

For the dry mass determinations, the seminal fluids were centrifuged, washed 3 times with distilled water and sperm smears prepared. The slides were immediately fixed either in alcohol-ether for 30 minutes or in 10% formalin for 5 minutes. After the alcohol-ether fixation, they were hydrated in a descending series of alcohol and carried into distilled water. All slides were mounted in distilled water and the coverslips sealed with paraffin. It should be pointed out that the dry mass determinations were the same with either of the fixatives mentioned above.

The dry mass of the sperm nuclei was determined with the Baker interference microscope using the $40\times$ and $100\times$ shearing objectives. The light source was an AH4 mercury vapor lamp and the 546 mμ line was isolated with Wratten filters 77A and 58. The interference bands of the microscope were spread according to

the instructions furnished with the microscope (Baker 1955). The phase shift was not determined by the usual *visual* matching technique between object and background, but by employing a photometric procedure[1], using the same photometric apparatus as in microspectrophotometry (POLLISTER and MOSES 1949, LEUCHTENBERGER 1950). Fig. 1 is a schematic diagram of the photometric apparatus in combination with the interference microscope used for dry mass determinations.

The method for determining the dry mass in a bull sperm was as follows: a sperm nucleus of normal cytological appearance is selected so that the immediate

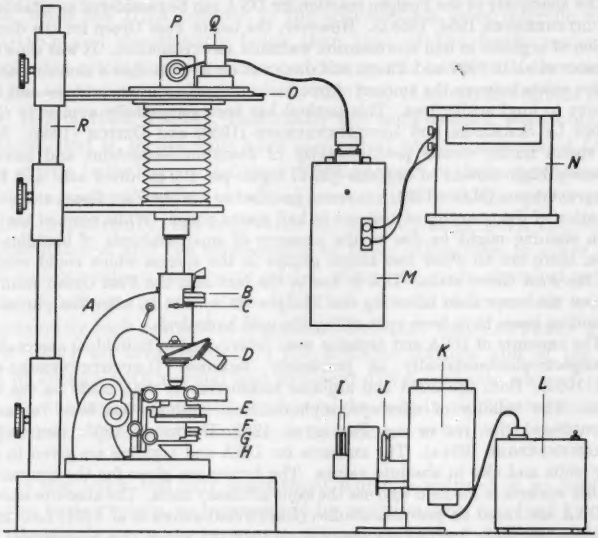

Fig. 1. Schematic diagram of Baker interference microscope with photometric attachments. *A* Baker interference microscope; *B* Rotating goniometer analyzer; *C* Quarter-wave retardation plate; *D* Shearing system objective; *E* Condenser; *F* Condenser diaphragm; *G* Polarizing plate; *H* Prism; *I* Wratten filters # 58 and 77 A; *J* Lamp diaphragm; *K* Lamp housing with AH 4 mercury vapor bulb; *L* Transformer; *M* Phototube power supply; *N* Galvanometer; *O* Upper diaphragm; *P* Phototube housing; *Q* Telescope; *R* Supporting stand

blank area surrounding it is free of 'ghost' images. These ghosts result from obtaining interference in shearing objectives. The sperm nucleus is centered with respect to the upper diaphragm, the long and short diameters of the sperms measured and the diaphragm closed to equal the short diameter of the sperm. Then keeping the same diaphragm opening, the slide is moved to an adjacent 'ghost' free blank area. The phototube is placed over this area. Now the goniometer is turned counterclockwise until the galvanometer shows a maximum deflection (100). At this point the goniometer reading is taken and recorded. In order to test whether this point is the maximum, the goniometer is turned clockwise about 25°. As shown

[1] We would like to thank Dr. HOWARD G. DAVIES of Kings College, London for his valuable suggestions and help in elaborating this photometric method.

in Fig. 2 the maximum goniometer reading was about 95 with a galvanometer deflection of 100, while at goniometer reading of 70, the galvanometer dropped to 85. After the maximum is established, the goniometer is turned counterclockwise in steps of 10° and for each goniometer reading, the galvanometer reading is recorded until a definite galvanometer minimum is obtained. Now the sperm nucleus is moved back into the field and the same procedure as outlined for the blank is used for the sperm nucleus. The curves of goniometer against galvanometer readings are plotted as shown in Fig. 2. It can be seen that the difference between maximum and minimum is 90° for the background as well as for the sperm nucleus. The differences in the goniometer readings between blank and sperm can be read directly from the two curves and represent the phase shift. It is obvious from the curve that the most favorable point to read the phase shift is about the midpoint between maximum and minimum, that is, when 45° are added to the minimum or subtracted from the maximum.

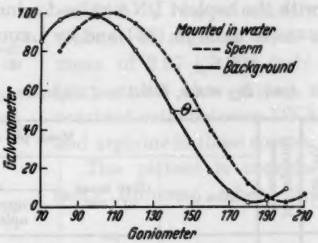

Fig. 2. Demonstration of phase shift in a bull sperm by interference microscopy (BAKER)

It should be mentioned that curves ought to be done every time a new biological object is examined for its dry mass. However, it is possible to obtain the midpoint readings by establishing photometrically only the maximum and minimum at the goniometer. The difference between maximum and minimum must of course be 90°, so that if 45° are either added to minimum or subtracted from maximum, the real midpoint is assured.

To compute the dry mass from the phase shift, the following data are required:
Θ = Phase Shift = difference between goniometer readings (cell structure reading-blank reading). This must be positive, otherwise goniometer was moved in wrong direction).

A = Area in square centimeters

Φ = Optical path difference (cell structure refractive index — medium refractiveindex) (thickness of cell structure).

X = Specific refractive increment: 0.18 for most biological materials.

λ = Wave length in centimeters.

Calculations

Calculation of Φ: Φ is simply $\frac{\Theta}{180} \times \lambda$; λ for mercury green light is 5.46×10^{-5} cm.

$$\text{Calculation of dry mass: the dry mass in grams} = \frac{A \times \Phi}{X}$$

Suppose $\Theta = 15$ as in Fig. 2 and the area of the sperm is 30 μ^2 or 3.0×10^{-7} cm^2.

$$\text{Then } \Phi \text{ will be } \frac{15 \times 5.46 \times 10^{-5}}{180} \text{ cm} = 4.54 \times 10^{-6} \text{ cm}.$$

$$\text{The dry mass will be } \frac{3.0 \times 10^{-7} \text{ cm}^2 \times 4.54 \times 10^{-6}}{0.18} \text{ cm that is}$$

$$7.6 \times 10^{-12} \text{ gr. or } 7.6 \times 10^{-9} \text{ mgm.}$$

Results

In Table 1 and Fig. 3, the data for area, dry mass, DNA and arginine of sperm nuclei are presented for 2 groups of bulls classified as fertiles and infertiles. Considering first the DNA values, it can be seen that the 15 fertile bulls show little variation from bull to bull. Each of the 15 bulls has a mean DNA content in the spermatozoa which is very similar to the mean DNA of 1.52 ± 0.004 (arbitrary units) a value which was computed on the basis of the *individual* DNA data found in 170 sperms (Fig. 3). This mean of 1.52 ± 0.004 is in very good agreement with the mean DNA found in 3000 spermatozoa from 55 other fertile bulls (Fig. 4) and also with the haploid DNA value for bulls previously reported by LEUCHTENBERGER et al. in 1951 and by LEUCHTENBERGER and SCHRADER in 1955.

Table 1. *Area, dry mass, DNA and arginine of sperm nuclei from 15 fertile and 15 infertile bulls*

Classification	Case No.	No. sperms measured	Mean per sperm ± standard error						DNA/arginine 10^{-3} mg.
			Area (μ^2)	Dry mass 10^{-9} mg.	DNA		Arginine		
					Arbitrary units	10^{-9} mg.	Arbitrary units	10^{-9} mg.	
Fertile	1	30	34.3 ± 0.44	7.6 ± 0.26	1.34 ± 0.08	2.68 ± 0.16	2.02 ± 0.08	2.02 ± 0.08	1.32
	2	25	34.3	7.3	1.38 ± 0.03	2.76 ± 0.06	1.96 ± 0.06	1.96 ± 0.06	1.40
	3	27	33.7 ± 1.09	7.4 ± 0.53	1.40 ± 0.03	2.80 ± 0.06	1.99 ± 0.04	1.99 ± 0.04	1.40
	4	30	32.5 ± 0.46	6.5 ± 0.15	1.41 ± 0.07	2.82 ± 0.14	1.97 ± 0.10	1.97 ± 0.10	1.44
	5	50	33.1 ± 0.44	7.6 ± 0.22	1.42 ± 0.04	2.84 ± 0.08	1.99 ± 0.06	1.99 ± 0.06	1.44
	6	30	34.0 ± 0.32	7.3 ± 0.32	1.44 ± 0.04	2.88 ± 0.08	2.12 ± 0.06	2.12 ± 0.06	1.34
	7	30	34.3 ± 0.63	7.0 ± 0.31	1.50 ± 0.03	3.00 ± 0.06	2.20 ± 0.09	2.20 ± 0.09	1.34
	8	28	31.8 ± 1.19	6.9 ± 0.49	1.54 ± 0.06	3.08 ± 0.12	1.91 ± 0.07	1.91 ± 0.07	1.62
	9	50	34.9 ± 0.47	7.4 ± 0.30	1.55 ± 0.05	3.10 ± 0.10	2.25 ± 0.06	2.25 ± 0.06	1.38
	10	30	36.0 ± 0.42	7.2 ± 0.33	1.59 ± 0.03	3.18 ± 0.06	2.10 ± 0.06	2.10 ± 0.06	1.52
	11	23	32.1	6.1	1.61 ± 0.04	3.22 ± 0.08	2.32 ± 0.06	2.32 ± 0.06	1.38
	12	23	31.8	7.3	1.62 ± 0.04	3.24 ± 0.08	2.03 ± 0.07	2.03 ± 0.07	1.60
	13	31	32.5 ± 0.37	6.8 ± 0.29	1.64 ± 0.05	3.28 ± 0.10	2.10 ± 0.03	2.10 ± 0.03	1.56
	14	30	34.2 ± 0.63	7.6 ± 0.33	1.64 ± 0.06	3.28 ± 0.12	2.05 ± 0.05	2.05 ± 0.05	1.60
	15	30	33.3 ± 0.45	7.0 ± 0.23	1.79 ± 0.04	3.58 ± 0.08	2.13 ± 0.07	2.13 ± 0.07	1.68
Infertile	1	52	37.2 ± 0.65	6.9 ± 0.35	{0.63 1.02 ± 0.03}	{1.26 2.04 ± 0.06}	2.92 ± 0.06	2.92 ± 0.06	{0.44 0.70}
	2	30	32.2 ± 0.68	7.2 ± 0.25	0.70 ± 0.03	1.40 ± 0.06	2.13 ± 0.06	2.13 ± 0.06	0.66
	3	39	33.5 ± 0.59	7.2 ± 0.26	0.89 ± 0.04	1.78 ± 0.08	2.05 ± 0.03	2.05 ± 0.03	0.86
	4	30	32.8 ± 0.45	7.7 ± 0.27	1.02 ± 0.09	2.04 ± 0.18	1.84 ± 0.07	1.84 ± 0.07	1.10
	5	30	32.4 ± 0.43	7.3 ± 0.14	1.04 ± 0.02	2.08 ± 0.04	2.22 ± 0.06	2.22 ± 0.06	0.94
	6	30	33.6 ± 0.54	8.1 ± 0.32	1.06 ± 0.04	2.12 ± 0.08	2.18 ± 0.05	2.18 ± 0.05	0.98
	7	25	32.0	6.9	1.08 ± 0.04	2.16 ± 0.08	2.02 ± 0.06	2.02 ± 0.06	1.06
	8	50	32.2 ± 0.54	7.9 ± 0.26	1.10 ± 0.03	2.20 ± 0.06	2.32 ± 0.04	2.32 ± 0.04	0.94
	9	30	33.0 ± 0.33	7.1 ± 0.27	1.14 ± 0.05	2.28 ± 0.10	2.11 ± 0.06	2.11 ± 0.06	1.08
	10	27	32.1 ± 0.28	7.3 ± 0.34	1.24 ± 0.07	2.48 ± 0.14	2.26 ± 0.04	2.26 ± 0.04	1.10
	11	30	34.1 ± 0.43	7.7 ± 0.21	1.25 ± 0.05	2.50 ± 0.10	2.38 ± 0.06	2.38 ± 0.06	1.04
	12	30	35.0 ± 0.31	7.6 ± 0.22	1.27 ± 0.09	2.54 ± 0.18	2.17 ± 0.06	2.17 ± 0.06	1.16
	13	25	31.6	6.6	1.27 ± 0.06	2.54 ± 0.12	2.57 ± 0.08	2.57 ± 0.08	0.98
	14	30	33.6 ± 0.64	7.8 ± 0.30	1.30 ± 0.07	2.60 ± 0.14	2.45 ± 0.05	2.45 ± 0.05	1.06
	15	30	34.6 ± 0.30	6.9 ± 0.20	1.32 ± 0.03	2.64 ± 0.06	2.55 ± 0.09	2.55 ± 0.09	1.04

In contrast to this constant haploid DNA content, the 15 bulls classified as infertiles show a considerable variation from bull to bull and in most instances, significantly lower DNA values. The mean DNA is 1.08 ± 0.004 for this group as compared with 1.52 ± 0.004 for the fertile group (Fig. 3).

Examining now the arginine data, it is evident that the fertile group with the constant amount of DNA also exhibits a very constant amount of arginine with a mean of 2.07 ± 0.006 (arbitrary units) thus giving a constant ratio between DNA and arginine in these sperms.

The picture is considerably different when the arginine data for the spermatozoa containing low amounts of DNA are examined. Here again the arginine values do not show a constancy, but vary from bull to bull and the mean arginine content is somewhat *higher* than 2.07, namely 2.28 ± 0.005. The difference between both groups is especially striking if the DNA/arginine ratios for the fertile and infertile groups are compared. It can be seen that in the fertile group, this ratio is consistently higher than the greatly varying ratios

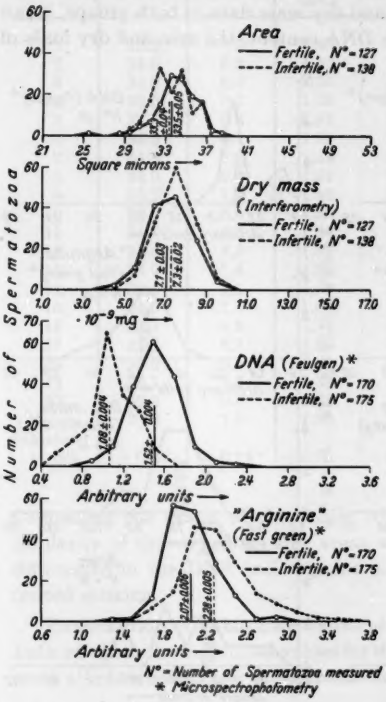

Fig. 3

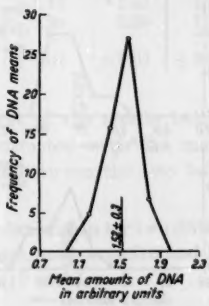

Fig. 4

Fig. 3. Area, dry mass, DNA and „arginine" content of 955 sperm nuclei from 15 fertile and 15 infertile bulls

Fig. 4. Mean amounts of DNA (Feulgen microspectrophotometry) in 3000 spermatozoa of 55 fertile bulls

in the infertile group. The DNA/arginine ratio for the fertile group of 1.47 is in very good agreement with the biochemical value of 1.48 reported by Vendrely and Vendrely in 1953 for bull sperms.

If we now look at the area and dry mass data in both groups, it can be seen that irrespective of the DNA content, the area and dry mass of

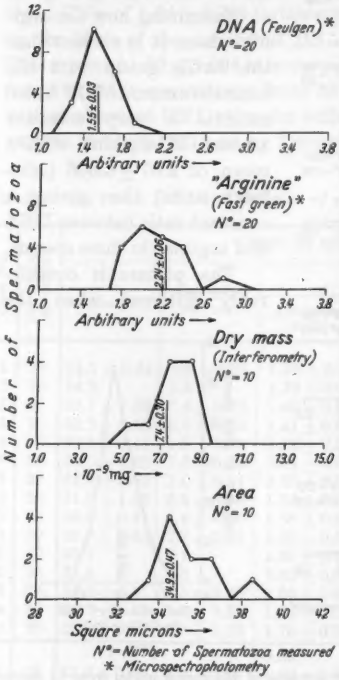

Fig. 5

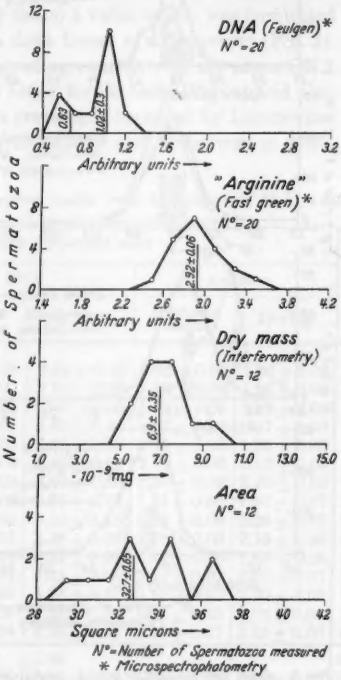

Fig. 6

Fig. 5. DNA, „arginine", dry mass and area of sperm nuclei from a fertile bull

Fig. 6. DNA, „arginine", dry mass and area of sperm nuclei from a sterile bull

all the spermatozoa examined are very similar. The dry masses are 7.1×10^{-9} mgm. ± 0.03 for the fertiles and 7.3×10^{-9} mgm. ± 0.02 for the infertiles. The areas are 33.3 μ^2 and 33.5 μ^2 respectively.

For further illustration of the differences between DNA and arginine and the similarity of the dry mass in the fertile and infertile bulls, the *individual* data on sperm nuclei are presented for 2 bulls in Figs. 5 and 6.

Table 2. *Determinations of area, dry mass, DNA and arginine in the same sperm nuclei*

Sperm No.	Area (μ^2)	Dry Mass 10^{-9} mg.	DNA		Arginine		DNA/ arginine 10^{-9} mg.
			Arbitrary units	10^{-9} mg.	Arbitrary units	10^{-9} mg.	
1	33.2	7.3	1.46	2.92	2.30	2.30	1.27
2	34.0	6.9	1.53	3.06	2.52	2.52	1.21
3	34.0	6.9	1.55	3.10	2.30	2.30	1.35
4	32.5	8.2	1.38	2.76	2.24	2.24	1.23
5	32.5	6.6	1.53	3.06	2.80	2.80	1.09
6	33.2	6.7	1.50	3.00	2.36	2.36	1.27
7	34.6	7.6	1.46	2.92	2.30	2.30	1.27
8	34.0	6.3	1.52	3.04	2.24	2.24	1.36
9	33.2	7.3	1.42	2.84	2.30	2.30	1.23
10	32.5	6.6	1.53	3.06	2.34	2.34	1.31
11	34.6	5.8	1.84	3.68	2.30	2.30	1.60
12	35.4	8.3	1.82	3.64	2.18	2.18	1.67
13	33.9	7.4	1.56	3.12	2.27	2.27	1.37
14	32.4	7.1	1.56	3.12	2.30	2.30	1.36
15	34.0	7.4	1.68	3.36	2.08	2.08	1.61
16	33.7	6.8	1.74	3.48	2.30	2.30	1.58
17	33.0	6.7	1.50	3.00	2.10	2.10	1.43
18	35.2	6.5	1.68	3.36	2.24	2.24	1.50
19	36.1	7.3	1.38	2.76	1.95	1.95	1.41
20	32.5	6.6	1.68	3.36	2.43	2.43	1.38
Means	33.8	7.0	1.56	3.14	2.30	2.30	1.37
Standard errors	± 0.24	± 0.14	± 0.03	± 0.06	± 0.04	± 0.04	± 0.04

Comparing the graph of the fertile with that of the sterile bull, the similarity of the dry mass and areas as contrasted with the marked differences in the DNA and arginine content between the two bulls is indeed striking.

Examination of Table 2 shows the same relationship for two different bulls in which the individual values for dry mass, area, arginine and DNA were all obtained in the same sperm nuclei (10 each).

Discussion

For a brief review, the results concerning the relationship between area, dry mass, DNA and arginine are presented in Table 3. Their

Table 3. *Means of separate mean values of area, dry mass, DNA and arginine in sperm nuclei from 15 fertile and 15 infertile bulls*

Classification	Area μ^2	Dry mass	DNA	Arginine	DNA/arginine
		10^{-9} mg.			
Fertile . . .	33.5 ± 0.33	7.1 ± 0.11	3.04 ± 0.07	2.07 ± 0.03	1.47 ± 0.03
Infertile . .	33.3 ± 0.36	7.3 ± 0.12	2.16 ± 0.10	2.28 ± 0.07	0.95 ± 0.05

absolute values are each a mean obtained from the 15 separate means given in Table 1 for the two groups. The findings may be summarized as follows:

1. Sperm nuclei with the constant and haploid DNA amount of 3.04×10^{-9} mgm. also have a constant amount of arginine and dry mass, and a constant size (Table 1, Fig. 3).

2. Sperm nuclei with a variable and deficient DNA content (Table 1, Fig. 3) with a mean of 2.16×10^{-9} mgm. have variable and somewhat higher arginine amounts but the dry mass and area are nearly identical with the normal ones (Table 1, Fig. 3).

Considering at first only the bull sperms of the fertile group, the agreement between the DNA and arginine data obtained by microspectrophotometric analysis on individual spermatozoa and the biochemical values published independently by the Vendrely's in 1953 is worth mentioning. Their biochemical values are 3.2×10^{-9} mgm. for DNA and 2.16×10^{-9} mgm. for arginine compared with the microspectrophotometric values of 3.04×10^{-9} mgm. and 2.07×10^{-9} mgm. respectively. Consequently their DNA/arginine ratio of 1.48 is practically identical with the ratio of 1.47 obtained in this study. This agreement between the data not only confirms the validity of Feulgen microspectrophotometry for quantitative DNA determinations but also establishes the usefulness of Fast Green microspectrophotometry for arginine in sperm nuclei.

Since the dry mass of sperm nuclei can be considered to consist mainly of DNA and protein, the protein content for sperm nuclei can be easily computed on the basis of the available data. The dry weight is 7.1×10^{-9} mgm., the DNA is 3.04×10^{-9} mgm. Consequently the protein equals 4.05×10^{-9} mgm. Since the arginine value was found to be 2.07, the protein in the bull sperm nuclei must be extremely basic in nature because its arginine content is 50%. The data actually prove the correctness of the Vendrely's assumption that „The bull sperm is not very different from that of trout and pike; hence the still unknown nucleoprotein, which might be obtained from bull sperm, if an appropriate method of extraction was available, would be a nucleoprotamine rich in arginine".

The conspicuously high arginine content of the proteins in the bull sperm nuclei is of special interest when one considers the fact that sperm nuclei consist almost exclusively of genetic material. If the genetic units in animals are made up not only by DNA as seems to be the case in bacteria and viruses (Avery et al. 1944), Hershey et al. 1953, Beadle

1955) but also by proteins (MAZIA 1954), the relatively simple protamine-like type of protein as found in the bull sperm nuclei is somewhat surprising. A more complicated type might have been expected for proteins contributing to genetic material. On the other hand, such proteins may be regarded as stable building blocks from which higher proteins can be synthesized. Furthermore the tight bond which such a basic protein can form with the nucleic acid may be a safeguard for the preservation of the genetic material. The constant ratio of DNA/arginine in all the sperm nuclei actually suggests a very close relationship and interdependence between the DNA and the protein.

Discussing now the bull sperms with the variable and deficient DNA contents, two findings deserve special consideration.

1. The finding that the dry mass of such sperms is the same as the dry mass of sperms with the normal haploid DNA content.

2. The finding that the variability of the DNA content is associated with a variability in the arginine content.

A priori, one would have expected that sperms containing lower amounts of DNA would also have a smaller dry mass than the sperms with the normal haploid DNA content. On the other hand, it should be kept in mind that for these studies only those spermatozoa were used for the determinations which had a normal cytological appearance. In other words, the shapes and sizes of the spermatozoa with normal and abnormal DNA content were nearly identical. This was confirmed by the fact that measurement of the sperm areas in both cases gave the same value, $33\,\mu^2$. Nevertheless one might argue that the sizes could remain unchanged, but the dry mass could be smaller due to the lower DNA content. This would be true provided the DNA was the only constituent which was decreased in quantity. Since there is no change in the dry mass, the unavoidable conclusion is that the protein in such sperms with low DNA must be larger in quantity than in sperms with the normal haploid DNA content. The variability of the arginine content showing a tendency towards higher values certainly supports this contention, but it would be desirable to be able to determine directly the total protein content in the sperms with a deficient DNA content.

Assuming the correctness of the concept that sperms with low DNA have an increased protein content, it would be of interest to examine the spermatogenic cells for their protein content. We know already that the DNA deficiency found in mature sperms originates during spermatogenesis as early as in the primary spermatocytes (LEUCHTENBERGER et al. 1954, LEUCHTENBERGER et al. 1955) and even before that in some

cases (Leuchtenberger and Schrader 1955). Does the same hold true for the proteins; that is, do the primary spermatocytes with the low DNA content already contain higher amounts of proteins than the primary spermatocytes with the normal DNA content? Some indication that this may indeed be the case comes from the observation that the sizes of primary spermatocytes with low and normal DNA contents are the same (Leuchtenberger et al. 1954). However it is realized that protein studies in spermatogenic cells are needed to establish this observation.

It might be worth while to mention that the sizes and dry mass of bull spermatozoa as reported in this study are practically the same as the ones obtained in ram spermatozoa with the Dyson interference microscope by Davies et al. in 1954.

Summary

Interferometric dry mass and microspectrophotometric DNA and arginine determinations were carried out on 4000 sperm nuclei from 85 bulls. The results obtained are as follows:

1. Spermatozoa containing a constant haploid amount of DNA (3.04×10^{-9} mg.) also have a constant arginine content of 2.07×10^{-9} mg. with a DNA/arginine ratio of 1.47.

2. Spermatozoa containing variable and low amounts of DNA also have variable and usually higher amounts of arginine with a ratio of 0.95 for DNA/arginine.

3. However the dry mass of sperm nuclei with an abnormal DNA content is nearly the same as the dry mass of sperm nuclei with the normal haploid DNA content. The dry mass for each group is 7.3×10^{-9} mg. and 7.1×10^{-9} mg. respectively.

4. On the basis of the DNA, arginine and dry mass data, a protein content of 4.06×10^{-9} mg. containing 50% arginine can be computed for normal bull sperm nuclei.

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